Collège Doctoral du Languedoc-Roussillon

THÈSE

Pour obtenir le grade de

Docteur

Délivré par l' **UNIVERSITE DE MONTPELLIER**

Préparée au sein de l'école doctorale
Sciences chimiques et biologiques pour la santé

Et de l'unité de formation et de recherche de médecine

Spécialité : **Reproduction humaine**

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EXPRESSION DES ARNm ET DES microARN DANS LES CELLULES DE CUMULUS HUMAINS : IMPACT DE L'AGE MATERNEL

Soutenue le 23 Septembre 2015 devant le jury composé de

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Acknowledgements

I address special thanks to Pr. Catherine Patrat and Pr. Paul Barrière for spending their precious time in reviewing my thesis manuscript and for their valuable suggestions. And, I am grateful to Pr. Christophe Beroud for his acceptance to participate in the jury committee.

Then, I address my thanks to Pr. Samir Hamamah for his admission to accomplish this thesis work in his lab and extreme thanks for the precious efforts of Dr. Ounissa Aït Ahmed who gave a lot for me to accomplish this thesis. I have a pleasure to thank Dr. Said Assou for his great and valuable contribution. Also, I express my gratitude to all peoples in the Institute for Regenerative Medicine and Biotherapy (IRMB) who helped me particularly Mme Monique Frei and Anne-Laure Courjaud, a training student. I would like also to thank the members of the postgraduate office in the Montpellier University and the doctoral school "CBS2" for their cooperation.

I gratefully acknowledge the higher education ministry of Iraq for giving me this opportunity and for the financial support, as well as university of Basrah, college of pharmacy, and all my friends and family for providing the facilities.

I must mention and dedicate this work for the souls of my dear parents who implanted the ambition and the volition in myself, and must not forget my beloved companions in this harsh and long way, my husband Majid Alheidary and my daughters Fatemah and Zainab, who suffered and hardly tolerated too much with me to finish this thesis, thank you all from profoundness of my heart.

Finally and firstly, before and after anyone, I would like to thank boundlessly the source of my strength and patience and the illumination who always illuminate my way, "my God" whose donations are greater than the thankfulness in all languages of the world.

Tamadir

Résumé

Expression des ARNm et des microARN dans les cellules de cumulus humains : impact de l'âge maternel

L'ovocyte se développe au sein d'un follicule, en contact étroit avec des cellules d'origine somatique, les cellules de cumulus (CC). Ces deux types cellulaires communiquent entre eux via des jonctions intercellulaires, permettant ainsi la régulation et la coordination du métabolisme pendant le développement et la maturation de l'ovocyte. Notre hypothèse est que l'expression et la régulation des gènes dans les CC joue un rôle crucial dans des fonctions essentielles pour la croissance de l'ovocyte et l'acquisition de sa compétence. Mes travaux de thèse comportent deux parties. Dans la première partie nous avons utilisé le séquençage haut débit pour examiner le répertoire des microARN (communément appelés miRNA) dans les cellules de cumulus et dans l'ovocyte. Les miRNA, séquences d'ARN non codantes dont la longueur varie entre 19 et 25 nucléotides, ont émergé récemment comme régulateurs majeurs de nombreux processus biologiques, dont le vieillissement. Nous avons identifié 32 miRNA spécifiquement dans les cellules de cumulus humains et seulement 3 dans l'ovocyte MII. Dans la seconde partie de nos travaux, nous avons analysé l'impact de l'âge maternel sur l'expression des gènes dans les cellules de cumulus. Alors qu'une baisse de la compétence de l'ovocyte avec l'avancement de l'âge maternel est bien établie, les bases moléculaires de ce phénomène demeurent peu connues. Dans une première étape pour aborder cette question, nous avons utilisé des puces à ADN pour analyser les profils d'expression des gènes des CC en fonction de l'âge maternel. De façon remarquable l'âge maternel impacte significativement l'expression de gènes qui sont critiques pour la maturation de l'ovocyte tels que les gènes impliqués dans l'angiogenèse, les voies de signalisation de TGF-ß et de l'insuline. Par l'utilisation d'outils bioinformatiques, nous avons aussi identifié des miRNA potentiels régulateurs de gènes impliqués dans des processus ou des voies impactés par l'âge ; ils pourraient constituer de nouveaux biomarqueurs pour prédire un vieillissement ovarien prématuré ainsi que la qualité et la compétence de l'ovocyte.

Mots clés : Ovocyte humain ; cellules de cumulus ; âge maternel ; transcriptome ; microARN ; séquençage haut débit ; microarray.

Abstract

Expression of mRNAs and microRNAs in the human cumulus cells: impact of maternal age

The oocyte develops in a follicle where it is in close contact with cumulus cells (CCs), of somatic origin. The two cell types undergo a bidirectional communication via gap junctions, which results in the regulation and coordination of the metabolism during oocyte development and maturation. We assume that gene expression and regulation in the CCs play a crucial role in functions that are essential for oocyte growth and competence acquisition. The present study may be subdivided in two parts. In the first part we used deep sequencing to investigate the repertoire of miRNAs in the cumulus cells and the oocyte. MicroRNAs that are noncoding RNA sequences whose length is approximately 19-25 nucleotides have emerged as important regulators in many biological processes including aging. Our data showed that 32 miRNAs were specifically expressed in human cumulus cells while only 3 miRNAs were identified in MII human oocyte. The impact of maternal age on gene expression in cumulus cells was addressed in a second part of my thesis work. While the correlation of oocyte competence decline with advancing maternal age is well established, little is known on its molecular basis. In a first attempt to address this issue, we used microarrays to study gene expression profiles of human cumulus cells according to maternal age. Remarkably, maternal age greatly impacted expression of genes that are critical for oocyte maturation such as genes involved in angiogenesis, TGF-β signaling, and insulin signaling pathways. Also, using bioinformatic tools, we identified miRNAs that potentially target some of the genes involved in the aging-impacted processes and pathways; this could candidate them as new biomarkers to predict premature ovarian aging and oocyte quality and competence.

Key words: Human oocyte; cumulus cells; maternal aging; transcriptome; microRNAs; deep sequencing; microarray.

CONTENTS

FIGURES

ABBREVIATIONS

8-OHdG	8-hydroxy-2'-deoxyguanosine
ACVR2A	Activin A receptor type IIA
ACVR2B	Activin A receptor type IIB
AND	Acide désoxyribonucléique
AFC	Antral follicle count
AGEs	Advanced glycation end-products
AGO2	Argonaute protein 2
ALCAM	Activated leukocyte cell adhesion molecule
AMH	Anti-Müllerian hormone
ANGPTL4	Angiopoietin-like 4
ARE	AU-rich elements
ARN	Acide ribonucléique
ARNm	Acide ribonucléique messager
ART	Assisted reproductive technology
ATP	Adenosine triphosphate
BCL2L11	B-cell CLL/lymphoma 2-like 11
BMP15	Bone morphogenetic protein 15
BMP6	Bone morphogenetic protein 6
BMPR2	Bone morphogenetic protein receptor type II
BMPs	Bone morphogenetic proteins
CAF1	CCR4-associated factor 1
cAMP	Cyclic adenosine monophosphate
CCR4	Chemokine (C-C motif) receptor 4
CCs	Cumulus cells
CDC25A	Cell division cycle 25 homolog A
cDNA	Complementary DNA
CGH	Comparative genomic hybridization
cGMP	Cyclic guanosine monophosphate
CKB	Creatine kinase brain
COC	Cumulus-Oocyte Complex
COS	Controlled ovarian stimulation
COX2 / COX-2	Cyclooxygenase 2/Cyclooxygenase-2
CTGF	Connective tissue growth factor
Cx	Connexin protein
CXCR4	Chemokines receptor 4
CYP17A1	Cytochrome P450, family 17, subfamily A, polypeptide 1
CYP19A1	Cytochrome P450, family 19, subfamily A, polypeptide 1
DCP1	Decapping protein 1
DCP2	Decapping mRNA 2
DGCR8	DiGeorge syndrome critical region 8
DNA	Deoxyribonucleic acid
DNMTs	DNA methyltransferases
E2	Estradiol
ECM	Extracellular matrix
EDC3	Enhancer of mRNA decapping 3
eIF4E	Eukaryotic translation initiation factor 4E
eIF6	Eukaryotic translation initiation factor 6

ESR1	Estrogen receptor 1
FC	Fold change
FDR	False discovery rate
FISH	Fluorescence in situ hybridization
FSH	Follicle stimulating hormone
FXR1	FragileX mental retardation protein 1
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GCs	Granulosa cells
GDF9	Growth differentiation factor 9
GDH	Glutamate dehydrogenase
Ge-1	Ge-1 ortholog
GJA1	Gap junction protein alpha 1
GnRH	Gonadotropin-releasing hormone
GPX3	Glutathione peroxidase 3
GREM1	Gremlin 1
GSTT1	Glutathione S-transferase theta 1
GW182/TNRC6-like protein	Trinucleotide repeat containing 6A gene- like protein
HAS2	Hyaluronic acid synthase 2
hCG	Human chorionic gonadotropin
HIG2	Hypoxia-inducible protein 2
hMG	Human menopausal gonadotropin
HMGA2	High Mobility Group AT-Hook 2
ICSI	Intra-cytoplasmic sperm injection
IGF-1	Insulin-like growth factor-1
INHA	inhibin
IRES	Internal ribosome entry site
IVF	In vitro fertilization
Kitl	Kit ligand
LH	Luteinizing hormone
LHR	Luteinizing hormone receptor
MAD2	Mitotic spindle assembly checkpoint protein
MGCs	Mural granulosa cells
MI	Meiosis I
miARN	microARN
MII	Meiosis II
miRISC	miRNA Induced Silencing Complex
miRNAs	microRNAs
mRNA	Messenger ribonucleic acid
mtDNA	Mitochondrial DNA
NCOR2	Nuclear receptor co-repressor 2
ORF	Open reading frame
OSGFs	Oocyte-secreted growth factors
PCK1	Phosphoenolpyruvate carboxykinase 1
PCR	Polymerase chain reaction
PDE3A	Phosphodiesterase 3A
PFKP	Phosphofructokinase platelet
PGS	Preimplantation genetic screening
piARN	"Piwi-interacting" ARN
piRNAs	Piwi-interacting RNAs
PRDX2	Peroxiredoxin 2

Pre-miRNA	Precursor miRNA
Pri-miRNA	Primary miRNA
PTEN	Phosphatase and tensin homolog
PTGS2	Prostaglandin-endoperoxide synthase 2
PTX3	Pentraxin 3
RCK/p54	DEAD (Asp-Glu-Ala-Asp) box RNA helicase 6
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPS6KA2	Ribosomal protein S6 kinase, 90kDa, polypeptide 2
RT	Reverse transcription
RT-qPCR	Reverse transcription quantitative polymerase chain reaction
SAM	Significance analysis of microarray
SAM-M	Significance Analysis of Microarray-Multiclass
SDC4	Syndecan 4
siRNAs	Small interfering RNAs
SIRT3	Sirtuin 3
SIRT5	Sirtuin 5
SMAD	Mothers against decapentaplegic
SMCB1	Structural maintenance of chromosomes protein 1B (SMC-1B)
SPSB2	SplA/ryanodine receptor domain and SOCS box containing 2
TGFβ	Transforming Growth Factor β
TNFAIP6	Tumor necrosis factor alpha-induced protein 6
TP53I3	Tumor protein p53 inducible protein 3
tRNA	Transfer ribonucleic acid
UTRs	Untranslated regions
VCAN	Versican
VEGF	Vascular endothelial growth factor
Xrn1	5'-3' exoribonuclease 1

Introduction

I- Normal ovarian function and age-related changes

A- Overview of follicle formation and oocyte maturation

Oogenesis and formation of the ovarian follicles (folliculogenesis) start in fetal life synchronously. Some oogonia begin conversion into primary oocytes, which enter the first meiotic division (meiosis I) at around 11–12 weeks of gestation. Oocytes of mammals develop inside structures called follicles and reach ovulatory maturity at puberty. A follicle consists of a primary oocyte covered by somatic follicular cells whose major functions are production of hormones and support the growth of oocytes capable to be fertilized.

The follicular development depends on paracrine and endocrine growth factors. The initial stages of this process are regulated independently of gonadotropins, however the final stages are known to be follicle-stimulating hormone (FSH) dependent. During the initial recruitment primordial follicles form in the fetus around midgestation. They are characterized by an oocyte arrested in prophase of meiosis I surrounded by one layer of flattened somatic cells. Then this layer transforms into a layer of cuboidal cells, herein the follicles are called primary follicles. In the secondary follicles (preantral follicles), oocyte grows and enlarges, the follicular cells proliferate to form many layers of granulosa cells, zona pellucida is formed, and the vascularization develops around the follicles. Then at puberty onset the follicles reach the antral stage and become dependent on FSH (beginning of cyclic recruitment). At this stage meiosis I resumes, after its completion the primary oocyte converts into a secondary oocyte; a fluid begins to accumulate between the follicle cells in the largest follicles forming one central cavity. Noticeably, only one or a limited number of antral follicles continues enlarging by accumulating more fluid to reach the preovulatory stage while others undergo atresia. Ovulatory follicle (17-20 mm of diameter) begins to bulge from the surface of the ovary and exerts a pressure on the outside of the ovary as well as the ovarian cells release enzymes in this region to digest the tissue at the weak point causing a rupture of ovary's surface. At last, the wall of the follicle breaks down and the mature oocyte with its surrounding cumulus cells (Cumulus-Oocyte Complex) is released from the ovary. This process is called ovulation. It occurs approximately once a month in women during their reproductive age, from puberty (11-15 years old) to menopause (45-55 years old). Just before ovulation, the inflammatory cells infiltrate into the area surrounding the follicle, which induces the ovulation as an inflammatory-like reaction. At this time the second meiotic division (meiosis II) takes place and arrests in metaphase to result MII oocyte added to the first polar body of meiosis I. This mature oocyte is surrounded by a thick layer of gel-like

material called zona pellucida and a layer of specialized granulosa cells called “cumulus cells”. The cells differentiate at the antral follicle formation stage and remain until after ovulation (Figure 1). After ovulation, the collapsed follicle forms the corpus luteum, which remains active for several months producing the progesterone needed for a successful pregnancy, when the oocyte is fertilized. In contrast, it disappears rapidly if fertilization does not occur.

The second meiosis resumes and is completed only after a spermatozoon penetrates into the mature oocyte, which results in the formation of an ovum after extrusion of the second polar body (23 chromosomes) (Chiras, 1999b; Goto et al., 2002; McGee and Hsueh, 2000; Palma et al., 2012; Uyar et al., 2013).

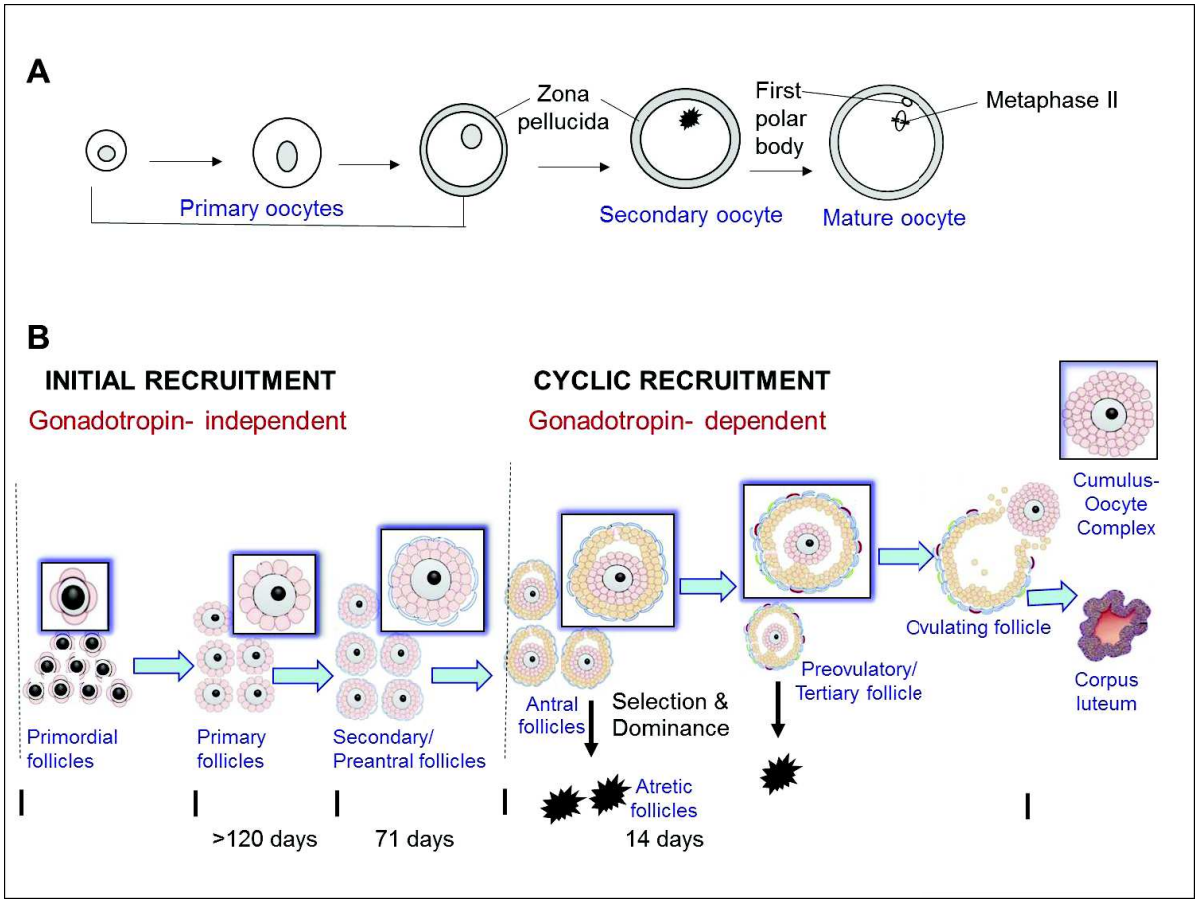

Figure 1: Schematic diagram of human folliculogenesis and oogenesis. (A) Oogenesis: Oocyte maturation synchronizes with follicular development. **(B)** In the human ovary, primordial follicles enter the growing phase through initial recruitment (Gonadotropin- independent growth) and then convert into primary follicles. Primary follicles need more than 120 days to reach the stage of secondary follicles, which requires 71 days of growth to reach the early antral stage. Thereafter the growth becomes dependent on gonadotropin (cyclic recruitment) and takes 14 days to get one dominant ovulating follicle whereas, the others undergo atresia (McGee and Hsueh, 2000) with modifications.

In human fertilization, many spermatozoa crowd around the ovulated oocyte and release enzymes from their acrosomes that dissolve the surrounding layers of the oocyte. After passing through the zona pellucida, the first spermatozoon to be in contact with the plasma membrane of the oocyte fertilizes it whereas all other spermatozoa are excluded. The fertilized mature oocyte becomes a zygote that undergoes rapid mitotic divisions and is soon converted into a morula, which differentiates into a blastocyst 5 days after fertilization, before implantation in the uterus. The blastocyst consists of the inner cell mass, which will become the embryo, and the trophoblast, a ring of flattened cells, which gives rise to the embryonic portion of the placenta (Figure 2). The embryo remains unattached in the uterus for 2-3 days. Then, implantation begins 7 days after fertilization when the blastocyst attaches to the uterine lining and digests its way into the endometrium (Chiras, 1999a).

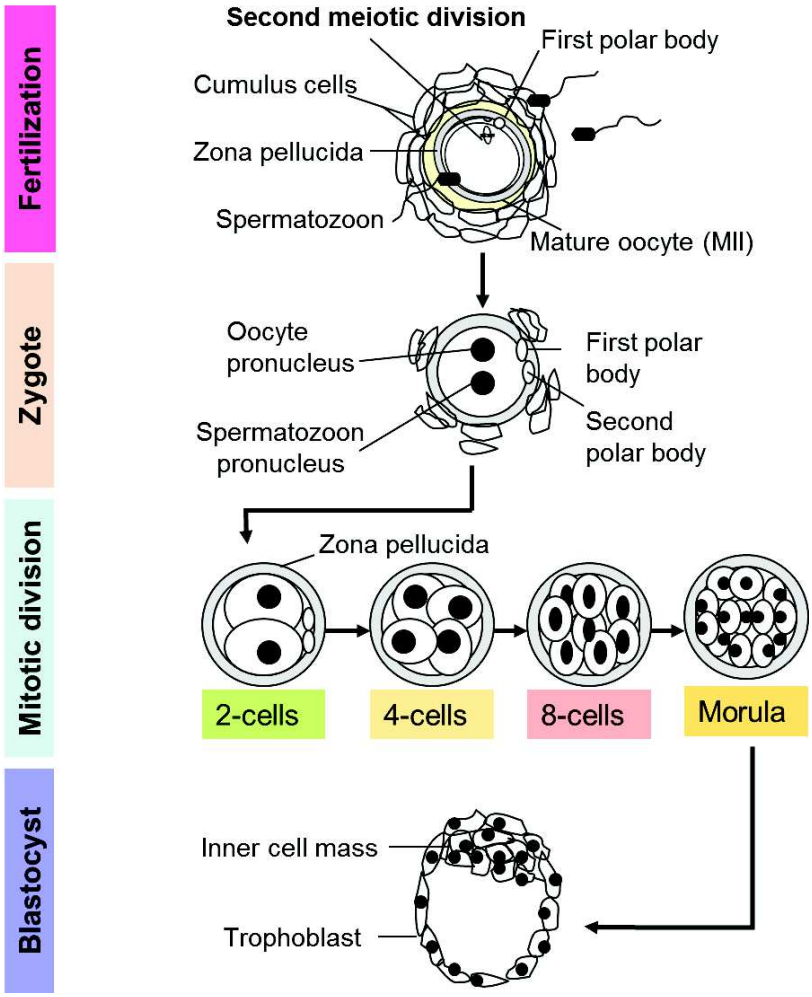

Figure 2: Schematic representation of the stages from fertilization to blastocyst. After fertilization the zygote undergoes mitotic division until reaching 16 cells, a stage that is called morula. Then the morula differentiates into a blastocyst and zona pellucida degenerates.

B- Hormonal regulation and aging-associated disorders

All events in the ovary are controlled by hypothalamus and pituitary gland via hormones such as follicle stimulating hormone (FSH) and luteinizing hormone (LH). These hormones act on oocyte maturation and then on ovulation. While FSH is crucial for follicles recruitment, LH represents an ovulatory hormone and regulates corpus luteum function. It is noteworthy that ovarian microenvironment plays an essential role in this control. Growth factors secreted from the oocyte itself or from the follicular cells, especially members of the Transforming Growth Factor β (TGFβ) superfamily including bone morphogenetic proteins (BMPs) and anti-Müllerian hormone (AMH), regulate FSH sensitivity. Hence, BMPs and AMH inhibit follicular prematuration and/or luteinization in small growing follicles by suppressing FSH action while in preovulatory follicles BMPs enhance FSH induced estradiol production. Noticeably, as shown in figure (3), the AMH production by the granulosa cells is induced by the oocyte at the beginning of follicle growth but it rapidly declines in the human antral follicles larger than 6–8 mm in diameter. Hypo or hypersensitivity to FSH is harmful to folliculogenesis (Anderson et al., 2012; Visser and Themmen, 2014). Thus, FSH sensitivity has a role in the follicle development and capacity to produce a mature oocyte capable to ovulate. Inhibin is another member of the TGFβ superfamily, which is synthesized by granulosa cells. It cooperates with activin to regulate follicle cells proliferation, steroidogenesis, gonadotrophin responsiveness, oocyte maturation, ovulation, and corpus luteum function. An increase of activin expression in the granulosa cells of immature follicles results in an increase of their proliferation and an induction of FSH secretion, which in turn suppresses androgen production. Activin also inhibits progesterone secretion. In contrast, inhibin level is low in the early follicular stages and rises in the preovulatory follicles to control proliferation of granulosa cells, repress FSH secretion, and enhance LH-induced androgen synthesis and luteal progesterone production (Knight and Glister, 2001). Other factors secreted from follicular cells such as sex steroids play important roles in the ovarian function maintenance by regulating folliculogenesis and contributing in the regulation of pituitary gonadotropin secretion when they are found in the ovarian microenvironment or the circulation respectively. Thereby, androgens including androstenedione, dihydrotestosterone, and testosterone (which converts into estradiol in the granulosa) have major actions during early stages of folliculogenesis through their receptors, which are highly expressed in cumulus cells. Moreover, androgens also induce proliferation of granulosa cells and follicular growth through the regulation of ovarian growth factors and increased gonadotropic sensitivity. Genes related to insulin-like growth factor-1 (IGF-1) and FSH receptors are

induced by androgens and are considered as important targets of androgen receptors. However, an excessive level of androgens leads to impairment of the ovarian follicular development (Gervasio et al., 2014).

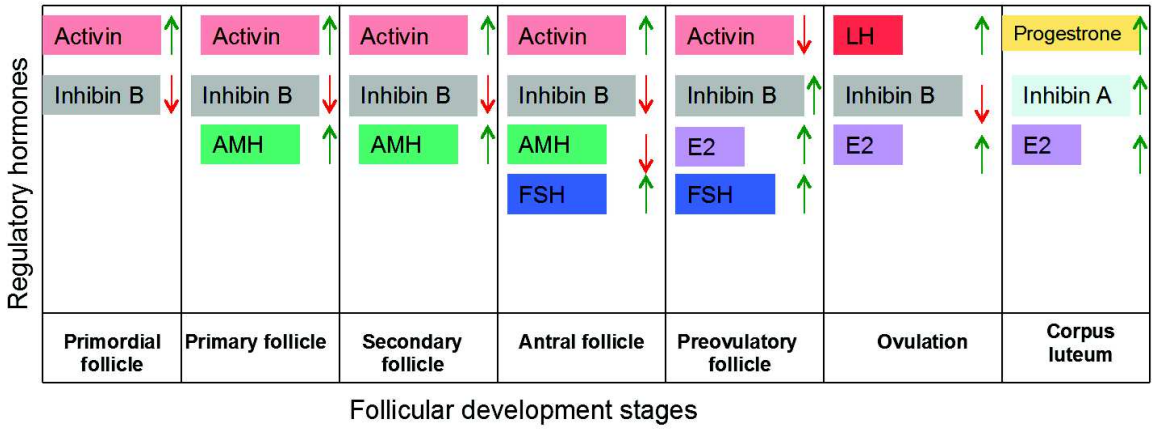

Figure 3: Production of regulatory hormones at different stages of follicular development. High hormone levels are indicated with the green arrow and low levels, with the red arrow.

Female fertility decline accelerates in women older than 37 years as a result of a decrease of oocyte quality and quantity. This is associated with a gradual increase of FSH and a decrease of AMH and inhibin B concentration levels in the blood circulation (Dayal et al., 2014; MacNaughton et al., 1992; te Velde and Pearson, 2002). The sex hormones that are secreted by the ovary (progesterone) and the pituitary gland (FSH, LH) relate with the menstrual cycle and the number of follicles in the ovary. Consequently hormonal disorders such as augmentation of FSH level, which happen with female aging result in menstrual cycle dysfunction and acceleration of follicle loss. Depletion of premature follicles with aging is associated with a decrease of inhibin B and AMH production by follicular cells of pre-antral and small antral follicles. This decrease of FSH negative regulators results in the production of high levels of FSH, which in turn minimizes the percentage of healthy oocytes for fertilization. This strong relationship among AMH, inhibin B, FSH, and antral follicle count (AFC) is highly significant for in vitro fertilization (IVF) as shown in previous studies (Ficicioglu et al., 2006; Li et al., 2012). Interestingly, these age-related hormonal changes are representative of menopause symptoms caused by ovary aging (Li et al., 2012; Tatone et al., 2008) and are serologic markers of ovarian stock (Nicopoullos and Abdalla, 2011). Further, low level of estradiol (E2) is another marker of the menopausal transition associated with the high level of FSH (El Khoudary et al., 2012) (Figure 4).

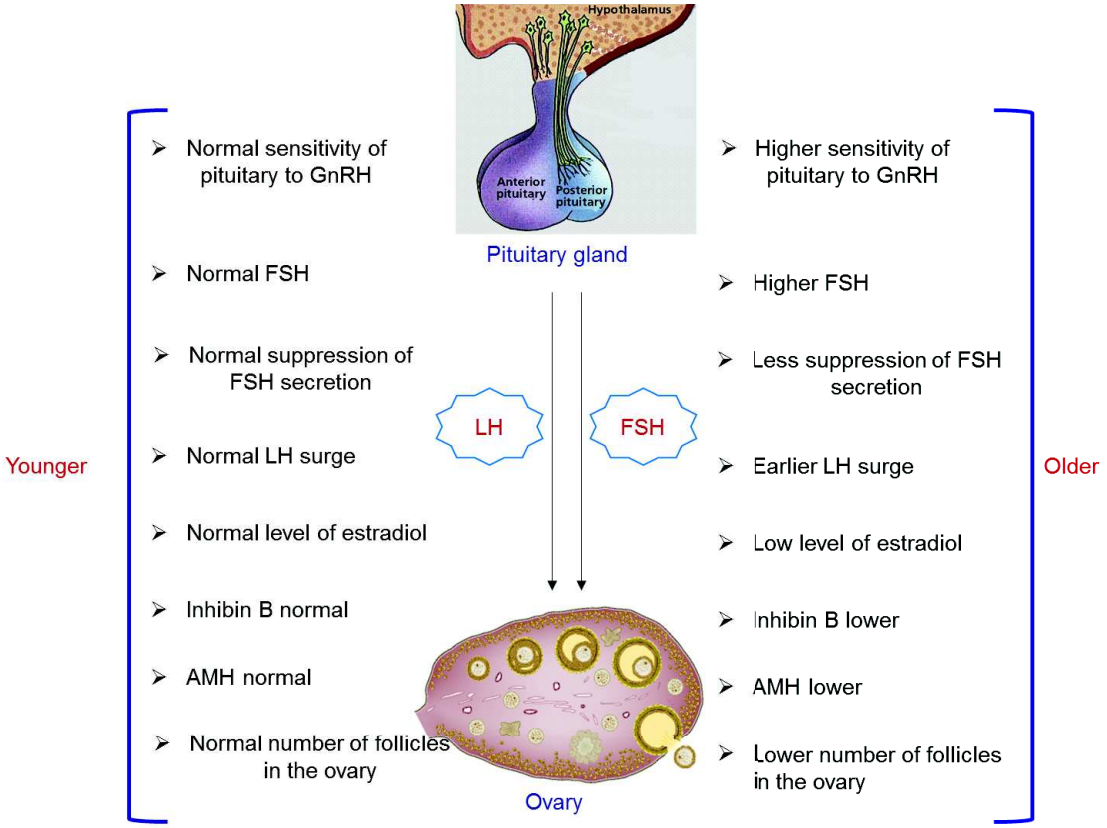

Figure 4: Reproductive hormones: relation to ovarian reserve and age-related changes. Hypothalamus and pituitary gland control ovarian events through FSH and LH. Aging leads to changes in these endocrine hormones, associated with accelerated depletion of follicles from ovarian pool and disorder of hormones that are crucial for follicle and oocyte development (Lambalk et al., 2009) with modifications. GnRH: Gonadotropin-releasing hormone.

C- Ovarian reserve and its age related decrease

There are approximately 1-2 million follicles in the ovary at birth, and 300 000-500 000 follicles at puberty. During the reproductive lifetime, only 400–500 follicles reach ovulation from 1000 primordial follicles, the others undergoing atresia (Li et al., 2012).

Women's reproductive success and ovarian function gradually diminish with aging (Bentov et al., 2011). Ovarian aging drives to a gradual decrease of the number of the primordial follicles that begins from the age of 31 and accelerates after 35. Approximately 25000 follicles remain at the age of 37. Finally, only around 1000 follicles reside in the ovaries around the age of 51 when menopause occurs (Li et al., 2012) (Figure 5).

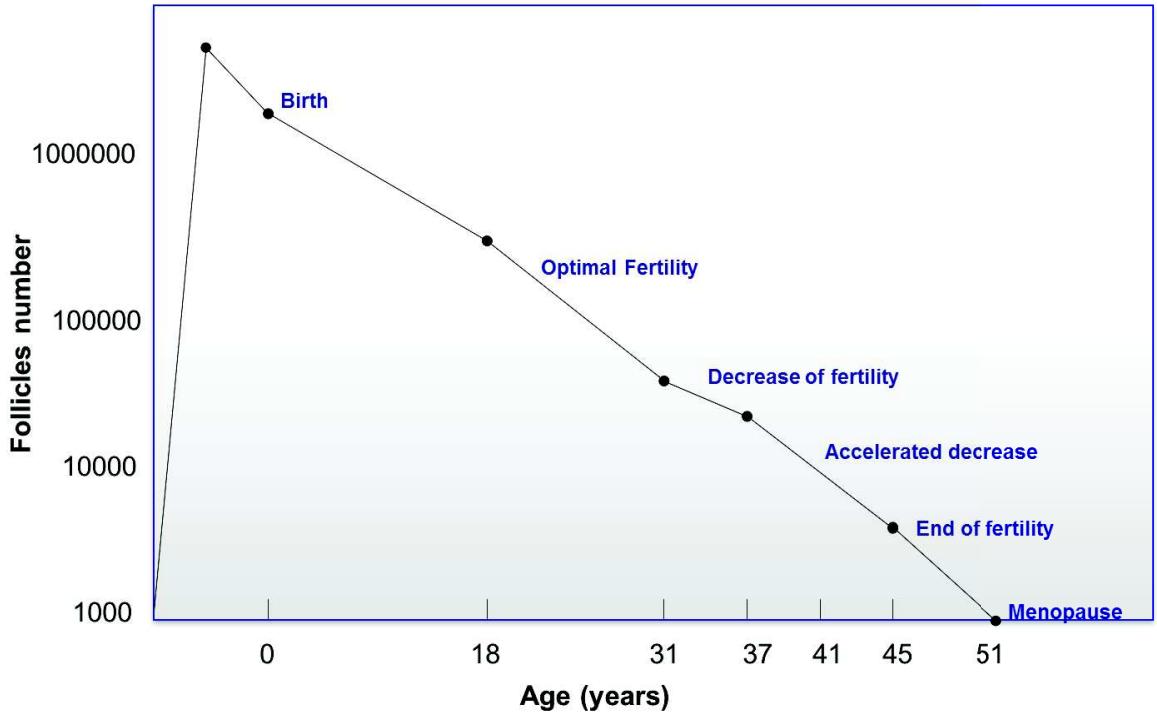

Figure 5: Decline of follicle number in pairs of human ovaries. The fertility is related with the number of follicles in the ovary. Optimal fertility is between the age of puberty and 30 years. While it decreases between 31- 37 years, this decrease accelerates until the age of menopause (51 years) (te Velde et al., 1998) with modifications.

D- Aged follicles

Advanced female age is a risk factor for infertility. In the old follicle, there are age-related changes in both intrinsic and extrinsic components that lead to reduced ability for producing competent oocytes.

1- Intrinsic components

1.1- Mitochondrial dysfunction

The number of mitochondria increases during oogenesis to provide ATP. At the time of formation of primordial follicles the number of mtDNA copies in the oocyte is about 10^3 and it reaches over 10^5 copies in the mature oocyte (Bentov et al., 2011). Mitochondrial oxidative phosphorylation of pyruvate supplies the primary source of ATP to the maturing oocyte. It is noteworthy that human oocytes with high level of ATP have a greater capacity to continue development and lead to better outcome (Dalton et al., 2014; Van Blerkom et al., 1995). This may explain why an oocyte that contains insufficient number of mitochondria during its maturation produces a poor quality embryo. Thus, oocyte competence relates with mtDNA copy number (Dalton et al., 2014).

Fragmented mtDNA may represent an indicator for oocyte senescence as it is more highly represented in older than younger oocytes (Keefe et al., 1995). Other cellular and

morphological abnormalities associated with intracytoplasmic mitochondrial aggregation have been shown in the aged oocytes of mice; this includes chromosome scattering, chromosome decondensation, cellular fragmentation, milky or dark cytoplasm, absence of nuclear/chromosomal DNA fluorescence and presence of cellular remains enclosed by the zona pellucida (Tarin et al., 2001).

Energy consuming processes such as spindle formation and function may be particularly sensitive to mitochondria number (Eichenlaub-Ritter et al., 2004). This probably accounts for the increase of aneuploidy rate in oocytes of older women as aging increases mtDNA mutations and deletions (Bentov et al., 2011).

1.2- Oocyte aneuploidy

Aneuploidy is extremely common in human oocytes, and is a major cause of congenital birth defects and miscarriages. Aneuploidy derives essentially from chromosome segregation errors that occur during oogenesis at meiosis I (Hassold et al., 2007). However, there are multiple mechanisms that may cause aneuploidy, of which two main mechanisms: (i) non-disjunction of entire chromosomes at meiosis I (MI) or meiosis II (MII) (Figure 6) (Zenzes and Casper, 1992) ; (ii) mal segregation of sister chromatids resulting from their premature segregation at MI; this mechanism may account significantly for human trisomy (Angell, 1991). Normally, the spindle checkpoint checks that the sister chromatids are properly attached to the microtubules. MAD2 is an essential component of the spindle checkpoint that plays a critical role at meiosis I by detecting a single, unaligned chromosome on the spindle. As result metaphase is delayed to allow the chromosomes to correct their position on the spindle and move properly. Down-regulation of MAD2 protein shortens meiosis I, increasing oocyte aneuploidy. Other protein such as cohesins that safeguard physical attachments between sister chromatids are essential at both meiosis I and meiosis II to prevent aneuploidies (Tatone et al., 2008). Interestingly, aging affects chromosome segregation through a loss of sister chromatid cohesion, hence the higher frequency of chromosomal aneuploidy in the oocyte with advanced maternal age (Fragouli et al., 2010; Nagaoka et al., 2012). The relationship of cohesion and checkpoint proteins with age-related aneuploidies is reinforced by the investigations on aged human and mouse oocytes that are MAD2 and SMCbeta1 deficient (Cukurcam et al., 2007; Steuerwald et al., 2001). These oocytes undergo a shorter meiosis and show increased premature segregation of sister chromatids (Tatone et al., 2008). This link between aneuploidy and aging is in relation to the follicular

microenvironment; so meiotic errors are due not only to intrinsic oocyte factors but probably also to the differences in follicular microenvironment (Van Blerkom et al., 1997).

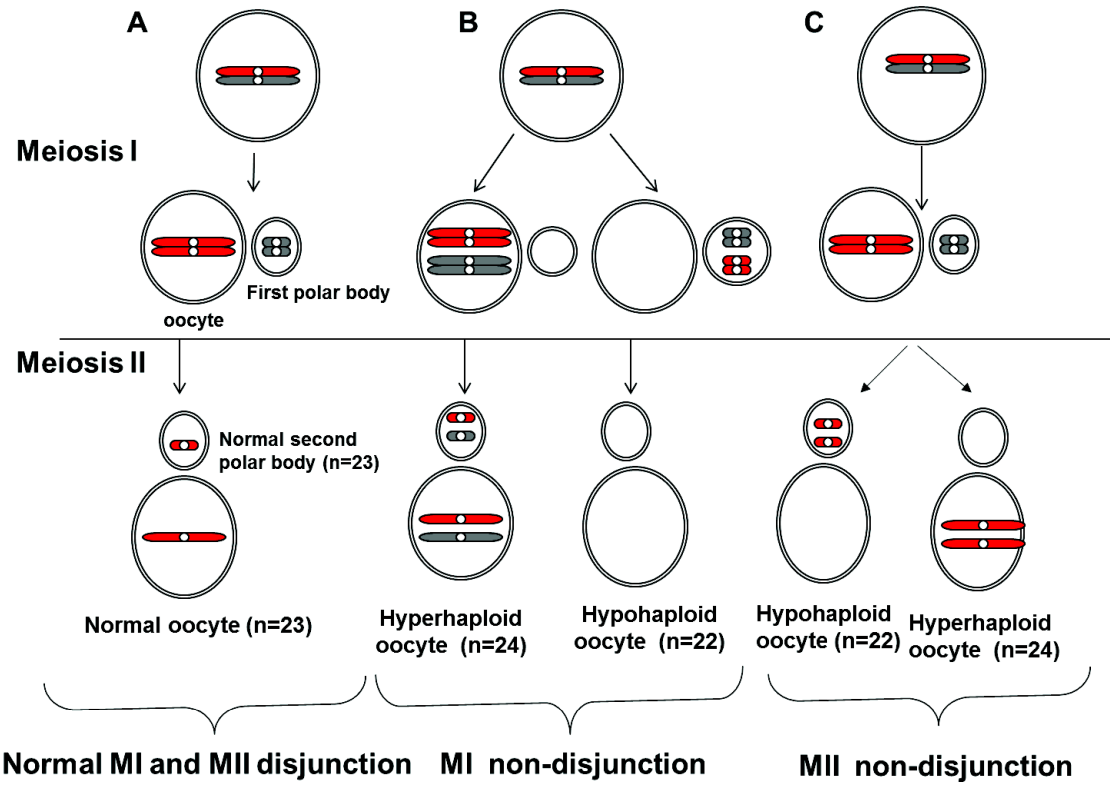

Figure 6: Meiotic divisions and chromosome non-disjunction. (A) Normal chromosome segregation during meiosis I and meiosis II results in haploid gametes (n= 23 chromosomes). Abnormal segregation of the chromosomes may result from the non disjunction of the homologues at meiosis I, MI **(B)** or the non disjunction of the sister chromatids at meiosis II, MII **(C)**, resulting in disomic or nullisomic gametes. Other missegregations events may occur that involve premature sister chromatid segregation resulting in hyperploidy or hypoploidy (Fragouli et al., 2011) with modifications.

2- Extrinsic components

It is proposed that a healthy microenvironment is crucial for follicular development and oocyte quality. In the aging ovary there are alterations in the ovarian microenvironment. As well as Pacella et al. (Pacella et al., 2012) demonstrated that the follicular environment and follicular cells function are altered according to female age.

2.1- Oxidative stress

Oxidative stress is recognized as an accelerator of aging, senescence or age-related changes (Harman, 2006). Although reactive oxygen species (ROS) has an essential role in follicle rupture during ovulation, excess ROS can also be responsible for oxidative stress and result in deleterious effects on mitochondria in the oocyte and granulosa cells (Behrman et al.,

2001; Van Blerkom et al., 1995). Therefore the balance between ROS and antioxidants within the follicle plays a critical role in oocyte maturation and granulosa cell functions. This homeostasis declines with aging, generating an imbalance, which leads to poor oocyte quality, an important factor of female infertility (Agarwal et al., 2012; Kirkwood, 2005). For instance impairment of antioxidant enzymatic defense leads to ovarian aging caused by higher oxidative stress damage (Li et al., 2012). An increase of oxidative stress in the follicular microenvironment with ovarian aging correlates with accumulation of advanced glycation end-products (AGEs) formed by non-enzymatic protein glycosylation and may lead to an altered follicular vascularization causing reduction of oxygen supply needed for follicular development (Tatone et al., 2008) (Figure 7). Further, one cause of IVF failure in older women is correlated with ROS level in follicular fluid (Wiener-Megnazi et al., 2004). For instance a high level in follicular fluid of 8-hydroxy-2'-deoxyguanosine (8-OHdG), a marker of DNA oxidation, is associated with failure of embryo transfer and pregnancy. When women with high levels of 8-OHdG are treated with antioxidants they show lower 8-OHdG concentration and higher pregnancy rate (Tamura et al., 2008).

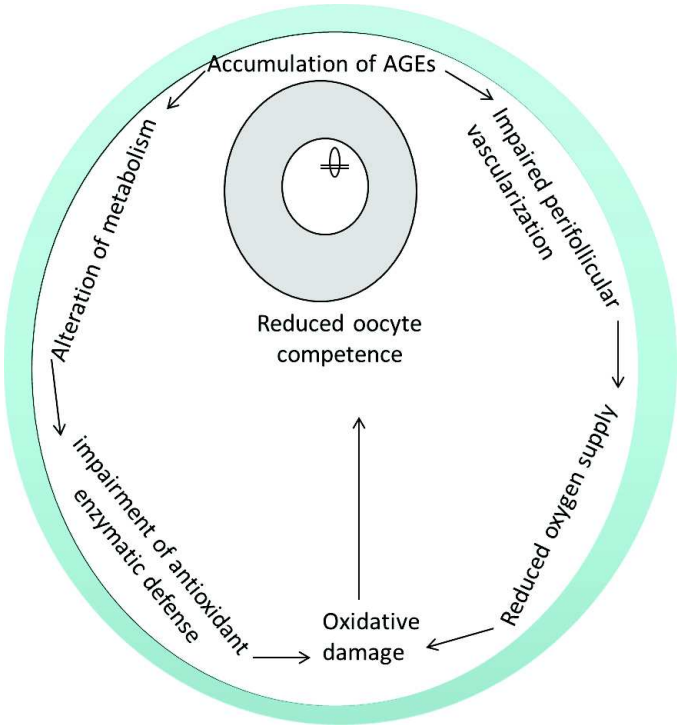

Figure 7: Possible mechanisms underlying the aging of follicular microenvironment. Gradual increase of AGEs (Advanced Glycation End-products) factors during the reproductive lifespan may lead to injury of blood and oxygen supply or harm of antioxidative defense that in the ultimate leads to reduced oocyte quality (Tatone et al., 2008) with modifications.

2.2- Hypoxia

Hypoxia is related with oxidative stress and occurs in living tissues when oxygen supply is not sufficient to cover the cellular metabolic demand (Lopci et al., 2014; Steiner et al., 2002). Hypoxia is an important process in aging. Importantly with aging the ovary acquires a different oxygen environment (Yeh et al., 2008). The decrease of oxygen supply to the growing follicle might be responsible for oocyte aging (Gaulden, 1992). The oocyte is dependent on oxygen diffusion via the surrounding granulosa cells and the follicular fluid (Friedman et al., 1997). In the follicular microenvironment, hypoxia plays an essential role in induction of angiogenic factors such as vascular endothelial growth factor (VEGF) in granulosa cells (Basini et al., 2004). While VEGF is crucial for folliculogenesis and formation of corpus luteum (Kaczmarek et al., 2005) its increase in follicular fluid of women with advanced reproductive age is consistent with hypoxic conditions within the ovarian follicles (Friedman et al., 1997). It may be considered as a compensatory attempt of granulosa and theca cells to modulate impact of hypoxia. However, this adaptive response is not sufficient to fully compensate for hypoxia (Tatone et al., 2008). Meiotic spindle abnormalities in the older oocytes may also be associated with elevated levels of VEGF (Klein et al., 2000). This is in line with the presence of disorganized meiotic spindles in oocytes isolated from severely hypoxic follicles (Van Blerkom et al., 1997). It is noteworthy that follicular oxygen content differs from one follicle to another in the same ovary (Van Blerkom et al., 1997). This intrafollicular oxygen variation may impact or determine oocyte competence (Van Blerkom, 1996); follicles with higher oxygen content (≥3%) having a better ability for oocyte development and fertilization (Li et al., 2012).

2.3- Aging of the follicle cells

Follicle aging is also characterized by the functional deterioration of granulosa cells, such as loss of mitochondrial activity, energetic failure, alterations in gene and protein expression profiles (Tatone et al., 2008). The impaired follicular oxidative phosphorylation and ATP production in women with advanced age is likely due to an increase of mutations in the follicular cells mitochondrial DNA (Seifer et al., 2002). Granulosa and cumulus cells from women of advanced maternal age exhibit increase of mtDNA deletions (Seifer et al., 2002), and alteration of the activity of the mitochondrial proteins such as sirtuin 5 (SIRT5), SIRT3, and glutamate dehydrogenase (GDH), a SIRT3 target (Pacella-Ince et al., 2014a; Pacella-Ince et al., 2014b). Moreover one can note a decrease of expression level of antioxidant enzymes, associated with age-dependent oxidative stress damage (Tatone et al., 2006). Further

observations on luteinizing granulosa cells show that aged women have fewer granulosa cells within each follicle as a result of increased granulosa apoptotic rate and decrease of their proliferation. Moreover a reduction of their steroids secretion is observed (Pellicer et al., 1994; Seifer et al., 1996). In addition to metabolic alteration in aged follicular cells (Pacella et al., 2012), the expression of 110 proteins is altered in human cumulus cells in relation to follicle aging (McReynolds et al., 2012). These age-related changes in follicular cells probably influence oocyte quality and competence, because of the interaction between the oocyte and the follicular cells (Gilchrist et al., 2008). Various studies have reported that obstruction of nutrients, proteins, and ions transfer between follicular cells and oocyte result in reduced oocyte competence (Buccione et al., 1990; Gershon et al., 2008; Sugiura et al., 2005). Moreover aged cumulus cells produce specific factors implicated in cellular senescence such as ceramide that result in accelerated apoptosis in the oocyte (Perez et al., 2005).

II- Gene expression in cumulus cells mirror oocyte potential

A- Cumulus cells: origin and communication with the oocyte

1- Origin of cumulus cells

At initiation of folliculogenesis, the oocyte of the primordial follicle begins to be surrounded by a flattened layer of somatic pregranulosa cells. These cells proliferate in relation to oocyte growth to form several cuboidal layers of granulosa cells. In the antral follicle, these cells differentiate into: (i) mural granulosa cells (MGCs), which are undifferentiated and form the inner pseudo stratified epithelial layers of the follicle; (ii) cumulus granulosa cells (CCs), which are organized in pseudo stratified epithelial layers, adjacent to the oocyte with which they communicate through gap junctions. Remarkably MGCs and CCs are different in their functions. MGCs have mainly a role in steroidogenesis whereas CCs undergo morphological and functional differentiation, which makes them capable of transferring metabolic molecules and regulators of meiosis to the growing oocyte (Buccione et al., 1990; Gilchrist et al., 2008). At the molecular level, CCs and MGCs also differ in their transcriptomes. More specifically CCs have higher expression of genes involved in metabolism and cell proliferation in comparison to MGCs which are enriched for transcripts encoding proteins involved in cell signaling and differentiation (Wigglesworth et al., 2015). The formation of cumulus cells during the preantral to antral follicle transition is required for the oocyte to mature and acquire its competence (Gilchrist et al., 2008), whereas cumulus expansion is required for

oocyte ovulation. Just before ovulation in response to LH stimulation CCs produce hyaluronic acid and expanded extracellular matrix leading to their expansion within cumulus-oocyte complex (Buccione et al., 1990). The expansion requires expression of four genes *HAS2*, *PTGS2*, *PTX3*, and *TNFAIP6* in addition to the presence of oocyte-secreted growth factors (OSGFs) particularly BMP15 and GDF9 (Gilchrist et al., 2008).

2- Communication between the cumulus cells and the oocyte

Folliculogenesis demands highly coordinated communication between the oocyte and the surrounding somatic cells, which is critical for oocyte maturation (Uyar et al., 2013). In Cumulus-Oocyte Complex (COC), oocyte regulates folliculogenesis and safeguards an optimal follicular microenvironment via secreting paracrine growth factors such as growth differentiation factor 9 (GDF-9), bone morphogenetic protein BMP-6, and BMP-15. These are the most important members of TGF-β superfamily for ovarian somatic cell function. They are required for hyaluronic acid synthesis, cellular proliferation, and cumulus expansion. They also play a role in preventing apoptosis in cumulus cells and luteinization in the growing follicles through inhibition of expression of the luteinizing hormone receptor (*LHR*) mRNA (Elvin et al., 1999; Gilchrist et al., 2008). GDF-9 can also regulate inhibin B production and Kit ligand (*Kitl*) expression in granulosa cells (Gilchrist et al., 2008; Shi et al., 2009). Kitl from granulosa cells of pre-antral follicles stimulates oocyte growth (Gilchrist et al., 2008), which probably accounts for folliculogenesis arrest in GDF-9-knockout mice (Dong et al., 1996). As a consequence of the bidirectional dialogue within the COC, several genes in CCs are induced by oocyte-secreted factors: for instance the hyaluronan synthase 2 (*HAS2*), tumor necrosis factor alpha-induced protein 6 (*TNFAIP6*), prostaglandin synthase 2 (*PTGS2*), and pentraxin 3 (*PTX3*), all being important for the synthesis of the cumulus extracellular matrix proteins (Elvin et al., 1999; Gilchrist et al., 2008). Alternatively, overexpression of the GDF9 receptor (*BMPR2*) in CCs leads to increase of the GDF9 action and activation of SMAD signaling. It is noteworthy that BMP15 and BMP6 also bind with BMPR2 (Assou et al., 2006; Gilchrist et al., 2008). Cumulus cells, in turn, support oocyte development by providing the molecules that are required for the oocyte growth including ions, metabolites, amino acids, and small regulatory molecules via gap junctions (Gilchrist et al., 2008; Gilchrist et al., 2004). Indeed, oocyte poorly metabolizes glucose and CCs are responsible for the majority of the cumulus-oocyte complex glucose and the energy (ATP) (Purcell et al., 2012). The gap junctions are long cytoplasmic extensions that penetrate the zona pellucida and are formed by connexin proteins such as Cx26, Cx30.3, Cx32, Cx37,

Cx40, Cx43, and Cx45 which have been identified in ovarian tissue of different species (Palma et al., 2012). CCs of humans use Cx43 (GJA1), which is essential to communicate with the oocyte and contribute to its competence (Feuerstein et al., 2007; Hasegawa et al., 2007). It is also through this channel that cumulus cells coordinate oocyte meiosis by passing cyclic guanosine monophosphate (cGMP) from cumulus cells into the oocyte. This results in an inhibition by cGMP of cyclic adenosine monophosphate (cAMP) hydrolysis by PDE3A phosphodiesterase. As a consequence the level of cAMP is high in the oocyte, which leads to meiosis blocking (Wigglesworth et al., 2013). Conversely LH, by reducing cGMP levels in CCs, causes a decrease of cAMP level in the oocyte and consequently meiosis resumption (Norris et al., 2009) (Figure 8).

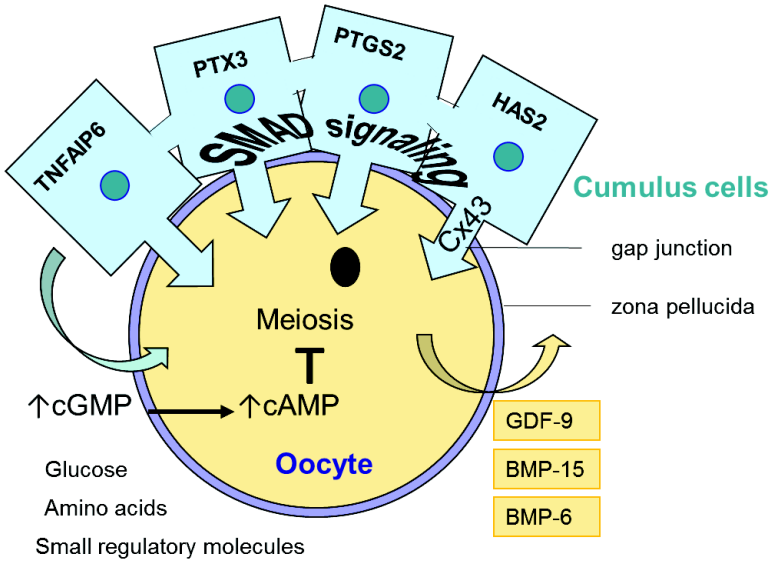

Figure 8: Cumulus and oocyte cross talk. Within cumulus-oocyte complex, oocyte communicates with its surrounding cumulus cells via cytoplasmic extensions called gap junctions, which penetrate zona pellucida for coordination between the two types of cells. Oocyte secretes paracrine growth factors such as GDF-9, BMP-15 and BMP-6 that play a critical role for follicular cell function. Cumulus cells, in turn, support oocyte nutrition and meiosis.

B- Gene expression profile of cumulus cells

1- Transcriptome

Transcriptomic is a valuable tool to study gene expression by using different techniques such as real-time PCR and DNA microarray. The measure of gene expression level by DNA microarray technology is based on hybridization of two differently and fluorescently labelled samples (test and reference samples) of cDNA probes to a high-density array of oligonucleotides that represent the target genes. The quantitative comparison of gene expression level in both samples is measured by the fluorescence at each spot equivalent to

the specific target gene on the array. Then, by using scanning confocal laser microscope the final microarray images are obtained and analyzed by different softwares (Duggan et al., 1999; van Hal et al., 2000; Xiang and Chen, 2000).

DNA microarray technology associated with bioinformatic analysis tools allow the simultaneous analysis of large numbers of expression data sets opening greater prospects for biomarker development. Study of gene expression profiles of cumulus cells and granulosa cells has allowed the identification of candidate biomarkers for (i) oocyte quality and competence (Adriaenssens et al., 2010; Cillo et al., 2007; Hamel et al., 2008), (ii) early embryo development (Anderson et al., 2009; McKenzie et al., 2004; van Montfoort et al., 2008) and (iii) embryo quality and pregnancy outcome (Assou et al., 2008; Hamel et al., 2010).

1.1- Follicular cell markers of oocyte competence and its developmental potential

The coordinated communication between the different follicular cell types during folliculogenesis is critical for the acquisition of oocyte competence that leads to success of fertilization and embryo development (da Silveira et al., 2012). Therefore, expression profile of follicular cells provides an indirect assessment of oocyte quality and developmental potential. In addition, the abundance and accessibility of cumulus cells lead many groups to use full transcriptome analysis of human cumulus and granulosa cells for prediction of oocyte competence (Hamel et al., 2008; van Montfoort et al., 2008). The expression levels of many genes in human CCs correlate with oocyte and/or embryo quality and serve as non-invasive biomarkers (Figure 9). For example high expression levels of *VCAN, PTGS2, GREM1*, and *PFKP* and *SDC4* underexpression in CCs predict high developmental potential of oocyte (Gebhardt et al., 2011). In other studies overexpression of *ALCAM, GREM1*, and *RPS6KA2* and underexpression of *PTGS2* were shown to significantly correlate with good embryo development (Adriaenssens et al., 2010) and up-regulation of *BCL2L11* and *PCK1* is significantly associated with embryo potential and pregnancy (Assou et al., 2008). In addition, the GDF-9-regulated CC-genes such as gremlin1 (*GREM1*), hyaluronic acid synthase 2 (*HAS2*), and pentraxin 3 (*PTX3*) are considered as markers of oocyte competence (Cillo et al., 2007). In contrast overexpression of the hypoxic indicators including glutathione peroxidase (*GPX3*) and chemokine receptor 4 (*CXCR4*) in human CCs has been shown to be associated with poor embryo quality (van Montfoort et al., 2008). Last, under-expressed CC genes (*SPSB2* and *TP53I3*) provide biomarkers of oocyte aneuploidy (Fragouli et al., 2012).

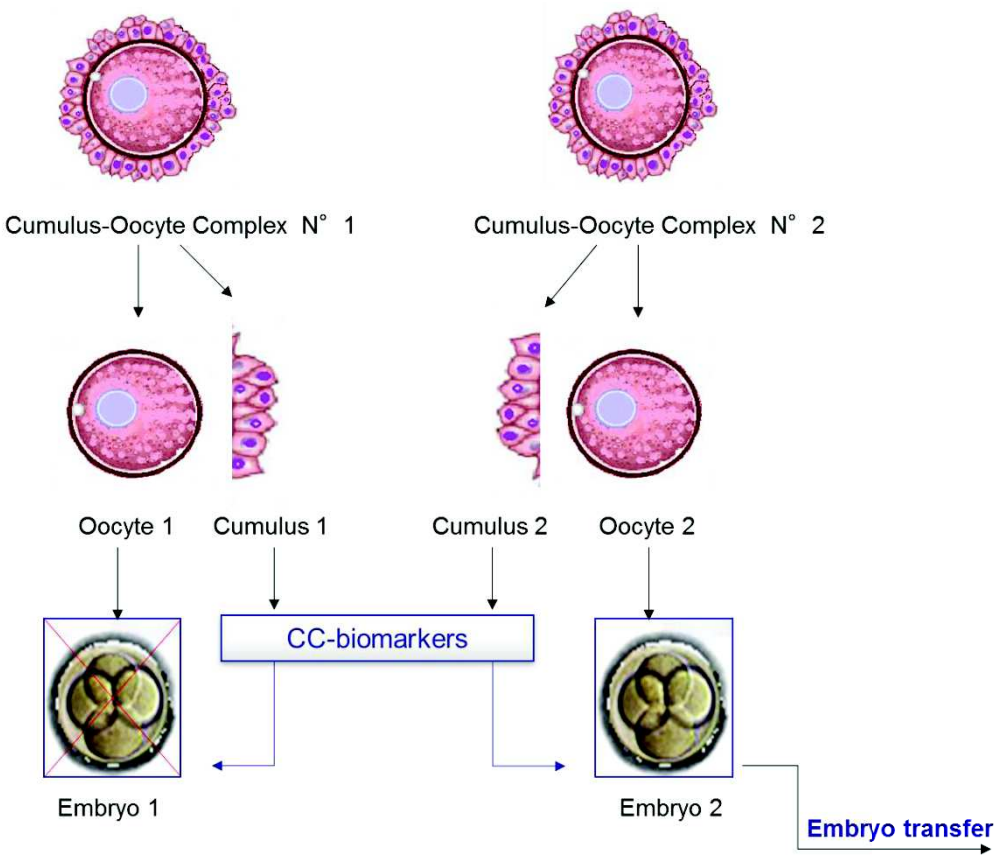

Figure 9: Cumulus genes express predictive biomarkers of oocyte competence. Differentially expressed genes in CCs can serve as biomarkers to select the competent oocyte that is able to give a good grade of embryo (Assou et al., 2008) with modifications.

1.2- Markers of the follicular cells in relation to female age

Little is known on the molecular signature of human follicular cells in relation to maternal age, however two studies reported biomarkers of oocyte developmental competence in aged follicular cells; indeed *CKB* and *PRDX2* overexpression in older CCs is associated with good embryo quality (Lee et al., 2010), while high expression of *GSTT1* in granulosa cells is a marker of oocyte quality decline and age-related infertility (Ito et al., 2008). In addition a negative correlation between embryo quality and increased apoptosis in cumulus cells from women aged over 40 years was reported (Lee et al., 2001).

2- MicroRNA regulation

In eukaryotes, gene expression is post-transcriptionally regulated by non-coding small RNAs including microRNAs (miRNAs), small interfering RNAs (siRNAs), and Piwi-interacting RNAs (piRNAs), through targeting 3′-untranslated region (3′UTR) of mRNA causing its degradation and/or inhibition of its translation (Watanabe and Lin, 2014). MicroRNAs

(approximately 19-25 nucleotides in length) have widely been studied as important regulators of diverse biological processes to control expression of many cellular proteins. Noticeably, 30-90% of mRNAs are regulated by miRNAs. A single mRNA may contain multiple binding sites for different miRNAs, miRNA in turn can target several mRNAs (Baley and Li, 2012). At the beginning of the 1990s, the first miRNA (*lin-4*) was discovered in *Caenorhabditis elegans* (Lee et al., 1993). The second miRNA to be discovered in *C. elegans* is (*let-7*) (Reinhart et al., 2000). At first both miRNAs (*lin-4* and *let-7*) were called small temporal RNAs before the microRNAs nomenclature came in 2001 followed by the description of more miRNAs in different organisms including humans.

Interestingly, the disruption of miRNA biogenesis and miRNA physiological roles, may lead to disease (Harvey et al., 2008). Quantitative analyses of miRNA expression in some tissues could provide reliable biomarkers to detect the type of disease particularly the types of tumor (Lages et al., 2011). Recently, a novel high-throughput RNA sequencing technology called deep sequencing technology has emerged to detect the differences in the expression level and structure of particular RNAs and to determine the sequence content and abundance of mRNAs, non-coding RNAs and small RNAs. Interestingly, bioinformatic analysis of sequence reads can provide both known and novel miRNAs (Pritchard et al., 2012).

2.1- Biogenesis of miRNAs

Small microRNAs biosynthesis begins within the nucleus. RNA polymerase II transcribes non-coding RNA into single strand RNA, which is processed into double strand hairpin RNA called primary miRNA (pri-miRNA). Then pri-miRNA is converted, into a ~70-100 base precursor miRNA (pre-miRNA) upon the action of Drosha enzyme and its RNA-binding cofactor (DGCR8). The pre-miRNA is then exported to the cytoplasm via exportin 5 to produce mature miRNA (double stranded miRNA) after further cleavage by enzyme Dicer 1. Thereafter, the mature miRNA loses one of its strands while the other loads onto the Argonaute proteins to search for complementary sequence of target mRNA and bind with its 3' UTR; this results in the formation of miRNA Induced Silencing Complex (miRISC), which suppresses gene expression. There is an alternative pathway for miRNA biogenesis; the miRNAs located within short introns (mirtrons) disbranch into pre-miRNAs after enzymatic splicing, before being transported to the cytoplasm (Baley and Li, 2012) (Figure 10).

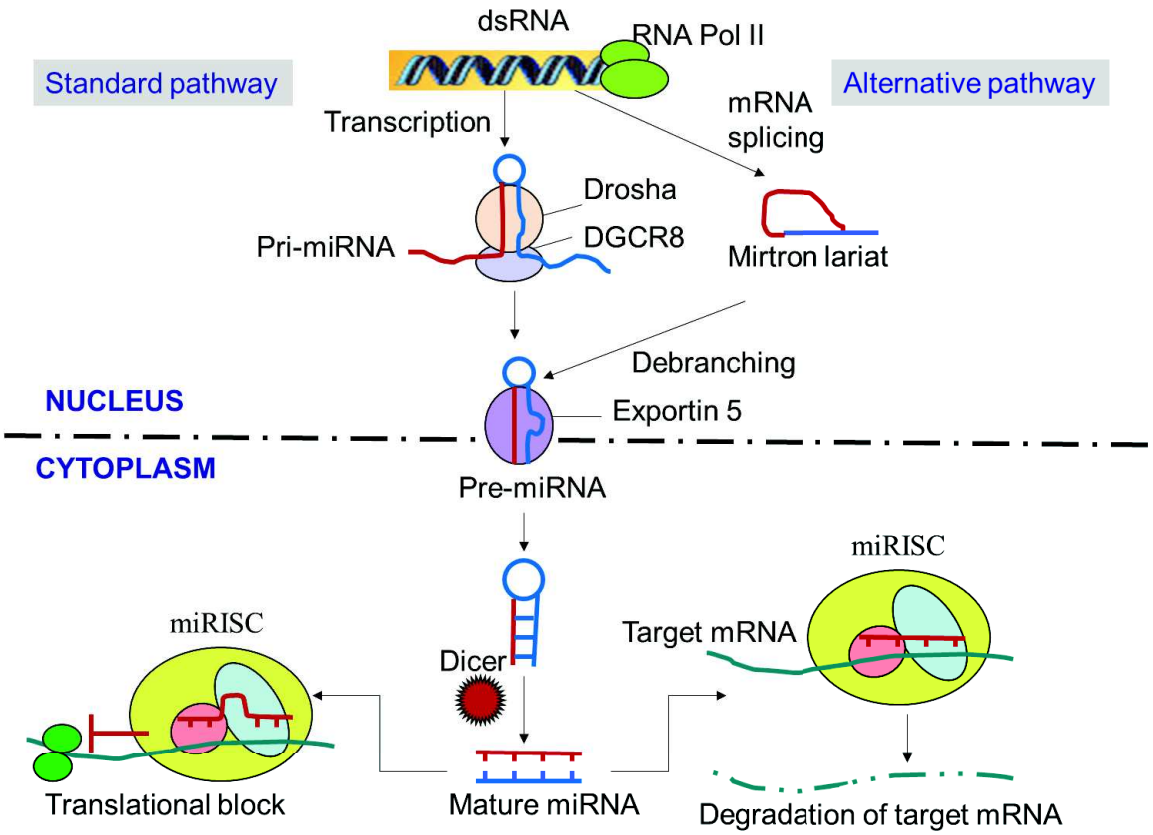

Figure 10: Model of miRNA biogenesis. There are two pathways (standard and alternative) for miRNAs biogenesis. MicroRNAs are transcribed in the nucleus by RNA polymerase II (Pol II) into long primary miRNA transcripts. Then they are cleaved into pre-miRNA by Drosha enzyme and its RNA-binding cofactor (DGCR8). Pre-miRNA is exported from the nucleus to the cytoplasm by exportin 5. In the cytoplasm, further processing by the enzyme Dicer produces a provisional miRNA duplex (mature miRNA). Only one strand of the miRNA duplex is integrated with Argonaute proteins to form miRISC by binding the mRNA 3' UTR, resulting in translation inhibition or decay of the mRNA target (Joshi et al., 2011) with modifications.

2.2- Mechanism of miRNA and mRNA interaction

The interaction of miRNA with its mRNA target occurs in the specific region of miRNA called seed region located between nucleotides 2 and 7 from 5' end to result in a perfect match (Lewis et al., 2003). The activity of miRNA depends on the size of the seed region. Moreover any mismatch in this region leads to a loss of miRNA interaction efficacy with its target particularly G:U type mismatch. Alternatively, when a mismatch occurs in the central region of the miRNA-mRNA duplex, it can affect the miRNA-mRNA interaction by inhibiting the endonucleolytic activity of AGO2 (Brennecke et al., 2005; Elbashir et al., 2001). In contrast, the miRNA-mRNA interaction is improved by the presence of an A and an A/U residues on the region of the mRNA corresponding to the position 1 and 9 respectively on both sides of the seed region (Lewis et al., 2005). Also, a complementary sequence at the miRNA 3′ end (especially between nucleotides 13 and 16) is necessary for the miRNA-

mRNA duplex stabilization (Grimson et al., 2007) (Figure 11). Further, the interaction of different miRNAs with the 3'UTR of the target increases its inhibition (Filipowicz et al., 2008). In addition to this canonical mechanism (miRNA binding with the 3'UTR of mRNA), some miRNAs have been reported to target genes by binding to their 5'UTR site or the open reading frame (ORF). This mechanism of miRNA intermediation is called non-canonical (Lytle et al., 2007).

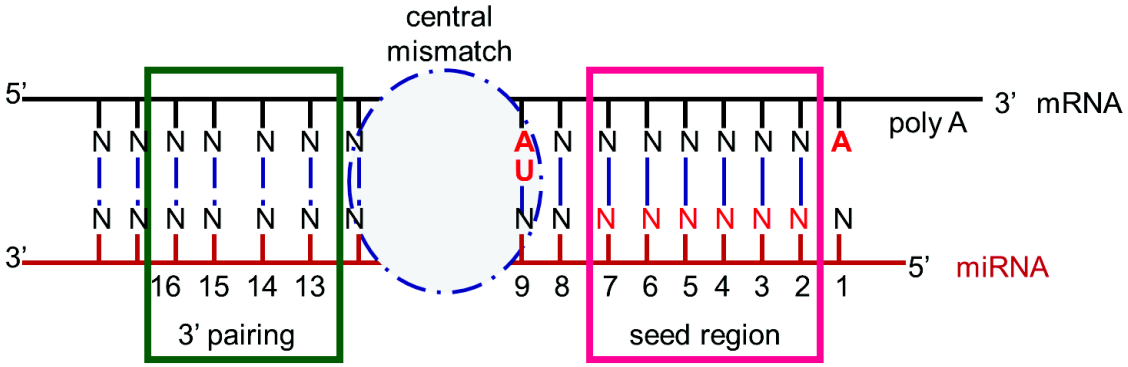

Figure 11: Schematic model of the miRNA interaction with the target gene 3'UTR. The perfect interaction between miRNA and its target is found in the seed region between nucleotides 2 and 7 from the 5′ end of the miRNA (pink rectangle). Other match needed for the miRNA-mRNA duplex stabilization could occur in the miRNA 3′ end (especially between nucleotides 13 and 16) (green rectangle). The efficiency of miRNA is increased in the presence of an A residue and/or an A or U residue on the mRNA corresponding to position 1 and 9 of the miRNA whereas, a central mismatch blocks endonucleolytic cleavage mediated by AGO2 (Lages et al., 2012).

Remarkably the target sites have different structural features (Figure 12). There are 3 types of sites: (i) canonical sites contain strong pairing and include three interacting sites (8mer, 7mer-m8, and 7mer-A1), which are all characterized by having 6 nucleotides for interaction located in the seed region (nucleotides 2-7), but they differ in the location of other interacting nucleotides. Thus, two other nucleotides in positions 1 and 8 for 8mer site whereas one additional nucleotide in position 8 and the A residue in position 1 for 7mer-m8 and 7mer-A1 sites respectively. (ii) Marginal sites have a weak pairing at the 5'end of the miRNA and consist of 6 nucleotides set within the seed region (this site is called 6mer), or between nucleotides 3 and 8 (called Offset 6mer). (iii) Atypical sites are more rare and contain a number of the interacting nucleotides at the 3'end compared with the other types of sites. They can have mismatch or perfect match at the miRNA 5' end (Lages et al., 2012).

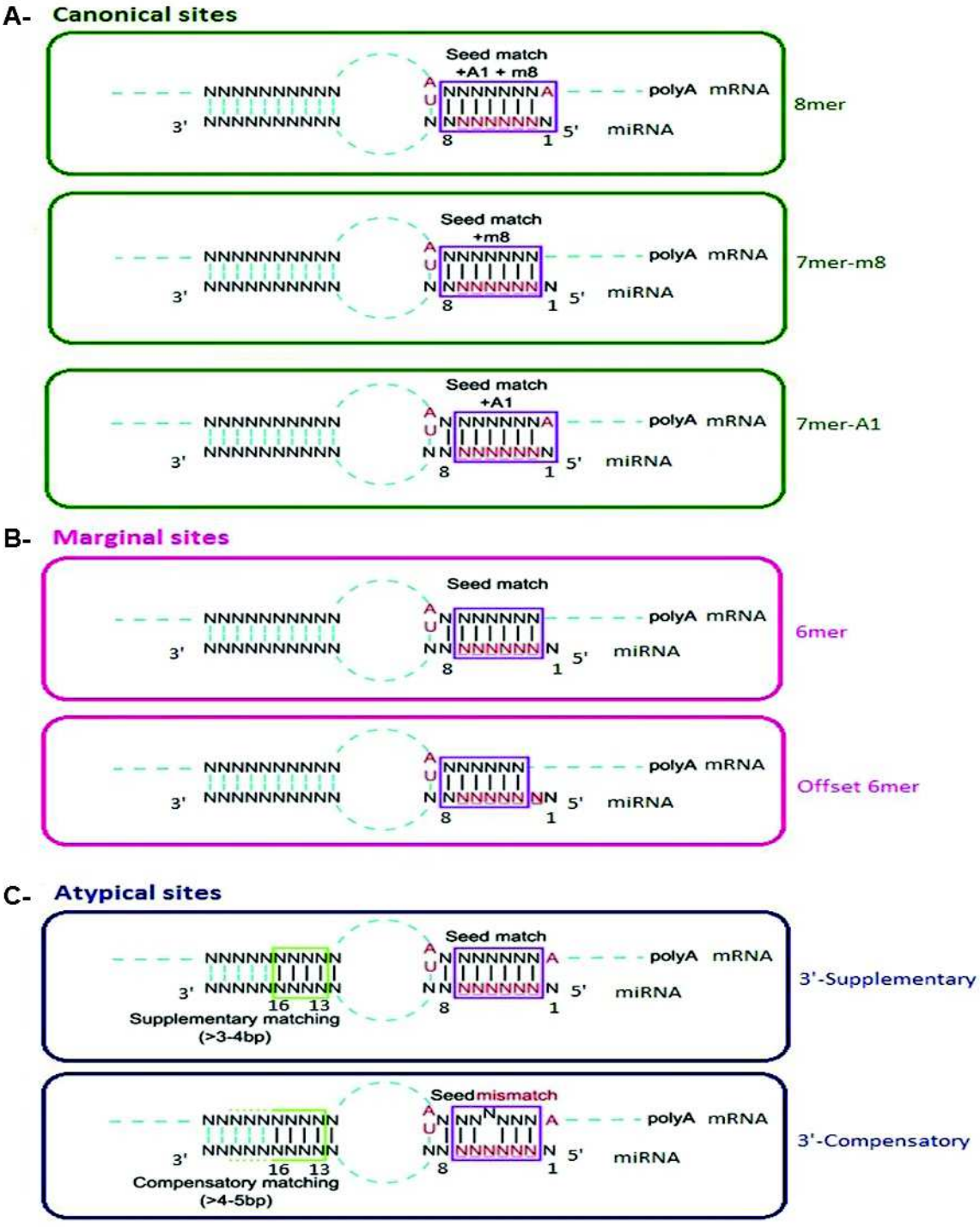

Figure 12: Different structural features for miRNA-mRNA interacting sites. (A) Canonical sites include three interacting sites (8mer, 7mer-m8, and 7mer-A1) composed of 7 or 8 nucleotides at the miRNA 5′ end (6 nucleotides in the seed region + position 1 and/or 8). **(B)** Marginal sites (6mer or Offset 6mer) consist of only 6 nucleotides in the seed region or 6 nucleotides between positions 3 and 8 respectively. **(C)** Atypical sites have a weak base-pairing in the 5′ region compensated by a number of the interacting nucleotides at the 3'end of miRNA (Lages et al., 2012).

2.3- Molecular mechanisms of microRNA-mediated gene regulation

The regulation of gene expression by miRNAs can be summarized in two main mechanisms which are suppression and activation of gene expression (Lages et al., 2012).

2.3.1- Gene expression regulation by suppressive mechanism

Suppression of gene expression is achieved by one of the following mechanisms:

(i) Inhibition of mRNA translation

Initiation, elongation, and termination are the three major steps of mRNA translation in the cytoplasm (Pestova et al., 2001). After transcription, mRNA processing results in two additional structures (cap m7G at the 5'end and poly A extension at the 3'end). Translation of mRNA initiates at the 5'end and cap m7G structure plays an important role in the regulation of translation initiation (Lages et al., 2012). Initiation of translation depends on ribosome recruitment either by involvement of cap-binding complex (cap and 5' end-dependent recruitment) or by an internal ribosome entry site (IRES) (Kean, 2003). Often, inhibition of translation by miRNAs takes place at the initiation step when the AGO2 proteins compete with cap-associated factors such as eIF4E for binding to the functional m7G cap structure of mRNAs (Kiriakidou et al., 2007). Also interaction of the miRISC with eIF6 factor can repress the initiation of translation by preventing the association of the 60S and 40S ribosomal subunits (Chendrimada et al., 2007) (Figure 13A). It is noteworthy that miRNAs are not able to inhibit mRNA translation at the initiation step when these mRNAs possess a nonfunctional cap structure or when they are translated from IRES (Humphreys et al., 2005). However miRNA can inhibit IRES-dependent translation at the post initiation step by ribosomal dropping off during the elongation, decreasing translational elongation speed, or degrading the newly synthesized polypeptide (Maroney et al., 2006; Nottrott et al., 2006; Petersen et al., 2006) (Figure 13B).

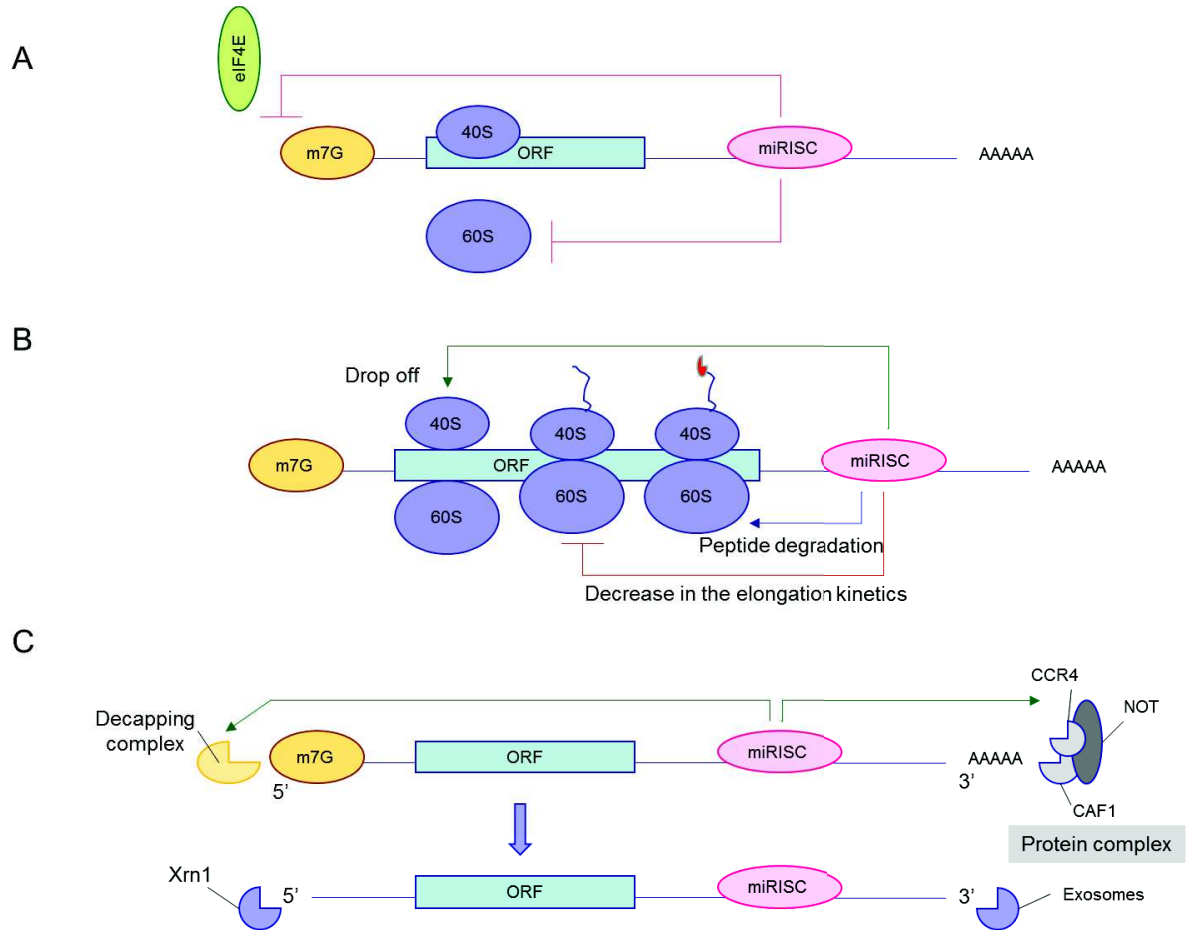

Figure 13: Mechanisms of gene expression repression by miRNA. (A) The repression occurs at translation initiation step by the competition of AGO proteins with eIF4E to prevent its fixation at the cap or by inhibition of the 60S ribosomal subunit recruitment. **(B)** Inhibition of translation at post-initiation step. There are three mechanisms (drop off, decrease in the elongation kinetics, and peptide degradation). **(C)** Degradation of mRNA, which can take place from 5′ to 3′ with the endonuclease Xrn1. This occurs after removal of the cap at the 5′ end by forming decapping complex or from 3′ to 5′ with exosomes after degrading the polyA tail through a complex of proteins (CCR4, CAF1, and NOT) that binds at the 3′ UTR of the mRNA (Lages et al., 2012).

(ii) Degradation of mRNA target

The suppression mechanism may also be achieved through miRNA-dependent reduction of mRNA stability. The mRNA stability relies on cap and poly A tail structures. Degradation of mRNA can occur from the two ends in the cytoplasm upon the action of exonucleases. The 5' to 3' degradation occurs after cap removal in the presence of Xrn1 exonuclease and protein complexes located in processing granule bodies whereas the 3' to 5' degradation is accomplished by protein complexes called exosomes (Figure 13C) (Lages et al., 2012). Additionally, miRNAs can also induce mRNA decay in the presence of GW182 proteins, which directly interact with deadenylase complexes (Braun et al., 2012).

2.3.2- Gene expression regulation by activating mechanism

Mostly, miRNAs negatively regulate mRNA expression. Nevertheless, the involvement of miRNAs in positive regulation of mRNAs is reported in specific cell types and conditions by activating their translation via AU-rich elements called ARE sequences at the 3'UTR of mRNAs. Also, in Drosophila, AGO2-miRISC mediates activation of gene expression by binding to eIF4E when the poly (A) tail is lost from the target mRNA. Moreover, AGO2 can associate with Fragile X mental retardation protein 1 (FXR1) in immature oocyte to activate mRNA translation by suppressing its down-regulation that results from the loss of AGO2 and GW182 protein interaction. Recently, some specific miRNAs have been shown to prevent the suppressive proteins to bind to their target sites, which results in up-regulation of gene expression (Lages et al., 2012; Valinezhad Orang et al., 2014).

2.4- MicroRNAs and ovarian function

Recently, miRNAs have been reported to play an important role in all biological processes that occur in the ovary including folliculogenesis, follicle atresia, luteal development and regression (Ling et al., 2014). Moreover they are involved in the regulation of the oocyte and CCs communication within the mammalian ovarian follicle (Assou et al., 2013a; da Silveira et al., 2012). MicroRNAs are also associated with hormonal regulation (Figure 14). On the one hand, significant up-regulation of miR-132 and miR-212 in mouse granulosa cells is induced by LH/hCG (Fiedler et al., 2008), on the other hand, production of progesterone, testosterone, and estradiol (E2) in the primary culture of human granulosa cells is affected by miRNAs (Sirotkin et al., 2009). Further, concentration of progesterone in serum of mice is increased by miR17-5p and let7b (Otsuka et al., 2008). Thus, miRNAs may govern the release of the main ovarian steroids. As shown in previous studies over-expression of miR-224 and miR-133b induces estradiol production in granulosa cells of mice via increasing *Cyp19a1* mRNA Levels (Dai et al., 2013; Yao et al., 2010), whereas, over-expression of miR-378 reduces the ability of granulosa cells to produce estradiol (Xu et al., 2011).

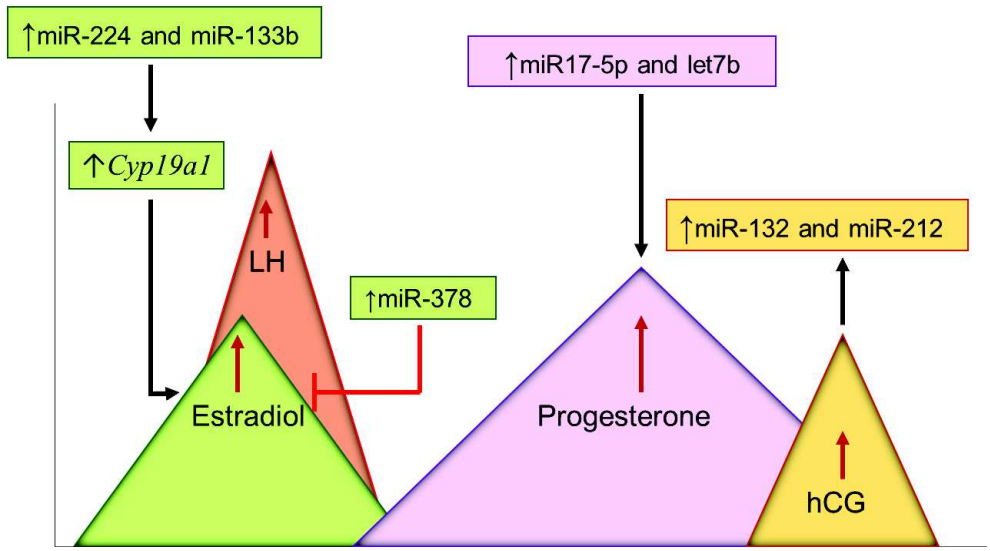

Figure 14: Schematic representation of miRNA role in the regulation of ovarian hormones.

2.5- MicroRNAs regulate follicular cells

It has been evidenced that primary action of miRNAs on ovarian function occurs via influencing ovarian somatic cells such as granulosa cells (Baley and Li, 2012). Accordingly, functional studies on somatic cells showed that inhibiting miRNA synthesis through deletion of Dicer 1 leads to blocking of development and infertility. Dicer 1 is essential for folliculogenesis, oocyte maturation, ovulation, and embryogenesis. Therefore, knockdown of Dicer in mice leads to defects in ovarian angiogenesis, follicle degeneration, and alteration in the expression of genes required for follicle and oocyte development such as *Amh*, *Inhba*, *Cyp17a1*, *Cyp19a1*, *Gdf9*, and *Bmp15* (Imbar and Eisenberg, 2014; Otsuka et al., 2008). Additionally, miRNAs also regulate proliferation and apoptosis of granulosa cells and then ovarian follicle development. For example miR181a suppresses expression of activin receptor II A (*Acvr2a*), affecting the proliferation of mouse granulosa cells (Zhang et al., 2013) whereas miR-224, which targets Smad family member 4 (*Smad4*) in the TGF-β pathway induces granulosa cell proliferation (Yao et al., 2010). The miR-224 also directly targets *Ptx3* leading to negative regulation of mouse cumulus expansion *in vitro* and ovulation *in vivo* (Yao et al., 2014). Also in mouse granulosa cells, miR-21 prevents apoptosis (Carletti et al., 2010) and promotes follicular cell survival during ovulation (Donadeu et al., 2012). Moreover, over-expression of *MIR23a* may play critical roles in regulation of human granulosa cells apoptosis through decreasing expression of X-linked inhibitor of apoptosis protein (Yang et al., 2012).

2.6 - MicroRNAs and ovarian aging

MicroRNAs have also been shown to regulate aging process and to be involved in many aging-related diseases in various tissues (Chen et al., 2010; Smith-Vikos and Slack, 2012). The role of miRNAs in ovary aging has been poorly investigated. Two studies on follicular fluid of mares and women report a possible role of miRNAs in ovarian aging (da Silveira et al., 2012; Diez-Fraile et al., 2014).

Results

I- Présentation résumée des résultats originaux en langue française

Mes travaux de thèse comportent deux parties. Dans la première partie nous avons utilisé le séquençage haut débit pour examiner le répertoire des microARN (miARN) dans les cellules de cumulus et dans l'ovocyte et dans une deuxième partie, nous avons analysé l'impact de l'âge sur l'expression des gènes et leur régulation par les miARN. Ces travaux sont rapportés dans deux articles originaux dont le contenu est résumé ici.

- Article 1

Assou S, **Al-edani T**, Haouzi D, Philippe N, Lecellier C-H, Piquemal D, Commes T, Aït Ahmed O, Dechaud H, Hamamah S. MicroRNAs: new candidates for the regulation of the human cumulus-oocyte complex (2013). *Hum Reprod 2013; 28, 3038-49.*

- Article 2

Female aging alters expression of human cumulus cells genes that are essential for oocyte quality. **Al-Edani T**, Assou S, Ferrières A, Bringer Deutsch S, Gala A, Lecellier C, Aït-Ahmed O, Hamamah S, (2014). *Biomed Res Int., 964614.*

A- Résumé des travaux présentés dans l'article 1

Le but de l'étude rapportée dans cet article a été (i) d'identifier et quantifier les petits ARN en particulier les miARN des cellules de cumulus humains et d'ovocytes MII, (ii) de caractériser les relations entre les profils d'expression des miARN et des ARNm de l'ovocyte et du cumulus. La méthodologie qui a été choisie est le séquençage profond suivi d'une analyse bioinformatique.

1- Séquençage des petits ARN de l'ovocyte et du cumulus

Le protocole expérimental est schématisé sur la figure 15. Le séquençage profond a été réalisé sur deux librairies de petits ARN d'ovocyte MII d'une part et de cumulus d'autre part. Un million de reads ont été réalisés. L'analyse bioinformatique révèle que 75% des miARN des CC et 31% des miARN de l'ovocyte ont une origine intronique alors que 56% des miARN de l'ovocyte ont une origine intergénique. L'analyse de la distribution des reads en fonction de leur taille met en évidence un pic à 19 nucléotides. Ce pic correspond précisément aux miARN. Un second pic est observé pour l'ovocyte MII à 25 nucléotides. Il correspond vraisemblablement aux "piwi-interacting RNAs" (piRNA) caractéristiques de l'ovocyte et dont la taille se situe entre 26 et 31 nucléotides.

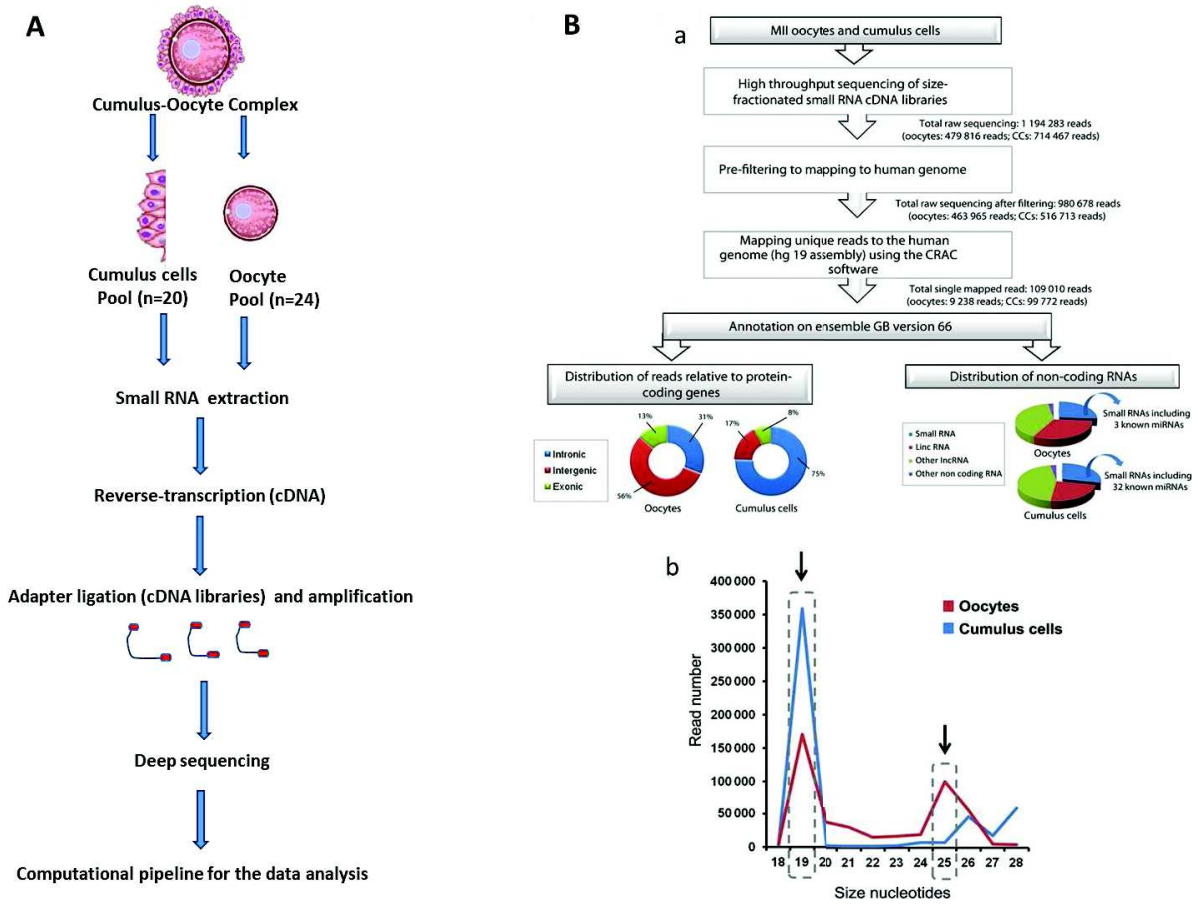

Figure 15 : Séquençage profond et analyse des miRNA de l'ovocyte et de cellules de cumulus humain. (A) Représentation schématique du protocole qui a été utilisé pour identifier les petits ARN dans les ovocytes MII et les cellules du cumulus (CC). **(B)** Représentation schématique de l'analyse des bioinformatiques des données. **(a)** Les « reads » issus du séquençage ont été filtrés puis alignés sur le génome humain de référence avec le logiciel de CRAC avant d'être annotés avec ENSEMBL (Ensembl API version 66). Les reads qui présentent un appariement parfait avec une localisation unique dans le génome sont classés selon leur localisation (intergénique, intronique ou exonique) d'une part et le type d'ARN non codant d'autre part (petits ARN dont les miRNA, lincRNA, lncRNA, autres ARN non codants). **(b)** Distribution des reads de la catégorie en fonction de leur taille. Il faut noter la présence d'un deuxième pic pour l'ovocyte qui correspond vraisemblablement aux piRNA.

L'analyse révèle la présence de 35 miARN précédemment identifiés, dont 32 dans le cumulus et 3 dans l'ovocyte (Figure 16A). Les miARNs les plus abondants dans les CC sont *LET7b* (51 reads), *LET7c* (31 reads) et *MIR21* (28 reads). Dans les ovocytes MII, les miARN les plus abondants sont *MIR184* (1988 reads) et *MIR10A* (555 reads). Afin de valider les données de séquençage, l'expression relative de cinq miARN pris au hasard a été analysée par RT-qPCR sur des pools indépendants d'ovocytes MII et de CC. Les résultats de qPCR sont en parfait accord avec ceux obtenus par le séquençage, validant ainsi ces données (Figure 16B).

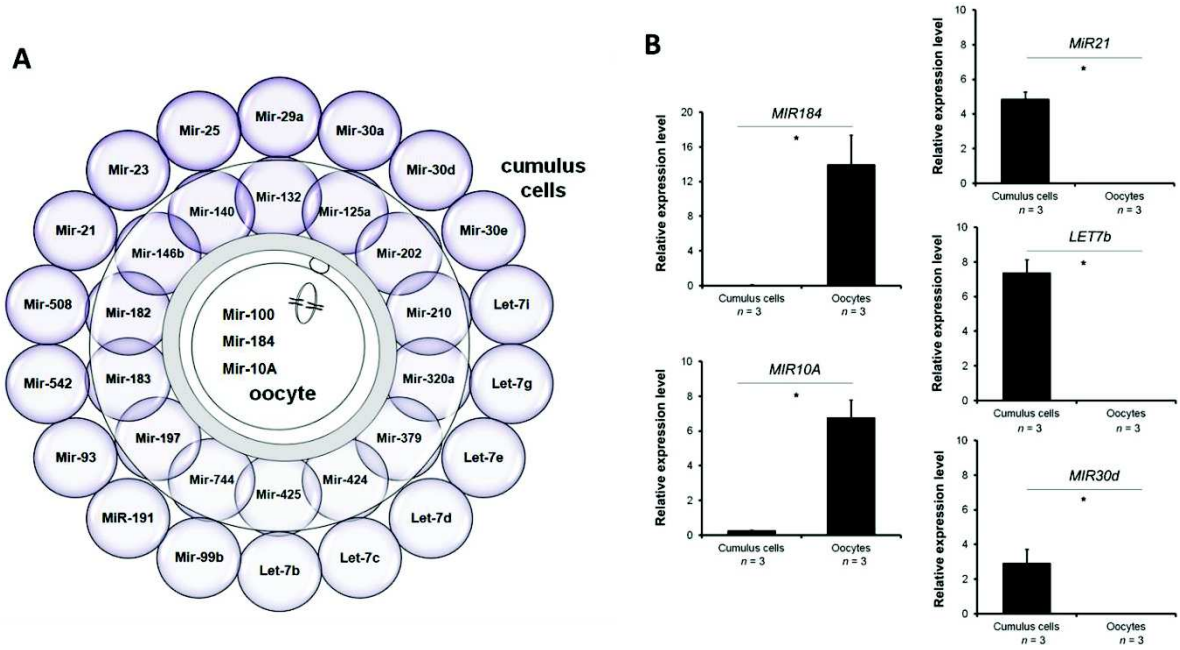

Figure 16 : miARN identifiés dans l'ovocyte et les CC. (A) miRNA identifiés par séquençage profond après application des critères définis plus haut. **(B)** Validation par RT-qPCR des niveaux d'expression de 5 miR pris au hasard, *MIR21, MIR30d, LET7b, MIR184* et *MIR10A*, sur des ARN de cumulus et d'ovocytes différents de ceux utilisés pour le séquençage. Les niveaux d'expression ont été calculés par rapport à l'expression de l'ARN de référence *RN U6-1*. La moyenne des échantillons ± SEM est représentée par des histogrammes. L'astérisque représente $p < 0,01$.

1.1- Identification des cibles des miARN

Afin d'identifier les cibles des miARN mis en évidence par séquençage, nous avons utilisé le logiciel GenGo Metacore.

1.1.1- Cibles des miARN enrichis dans l'ovocyte

Ce travail nous a donc permis de mettre en évidence 30 ARNm cibles validés expérimentalement pour les 3 miARN (*MIR184*, *MIR100* et *MIR10A)* identifiés dans l'ovocyte MII. *SMARCA5* (*SWI/SNF*-related matrix associated actin-dependent regulator of chromatin, subfamily A member 5) a été décrit comme cible de *MIR100* (Bhushan and Kandpal, 2011). Or ce gène est surexprimé dans l'ovocyte comme dans les cellules souches pluripotentes, faisant de lui un candidat dans la reprogrammation de la chromatine (Assou et al., 2009). Quant à *MIR184,* il a pour cible *NCOR2* (nuclear receptor co-repressor 2), un médiateur de la répression transcriptionnelle de récepteurs nucléaires (Wu et al., 2011). Finalement *MIR10A* est connu comme régulateur de *HOXA1,* un gène à homeobox dont l'ARNm est abondant dans l'ovocyte (Figure 17A) (Lund, 2010). Il faut souligner

l'importance de *HOXA1* dans la régulation de l'expression de gènes spécifiques de l'ovocyte de souris (Huntriss et al., 2006).

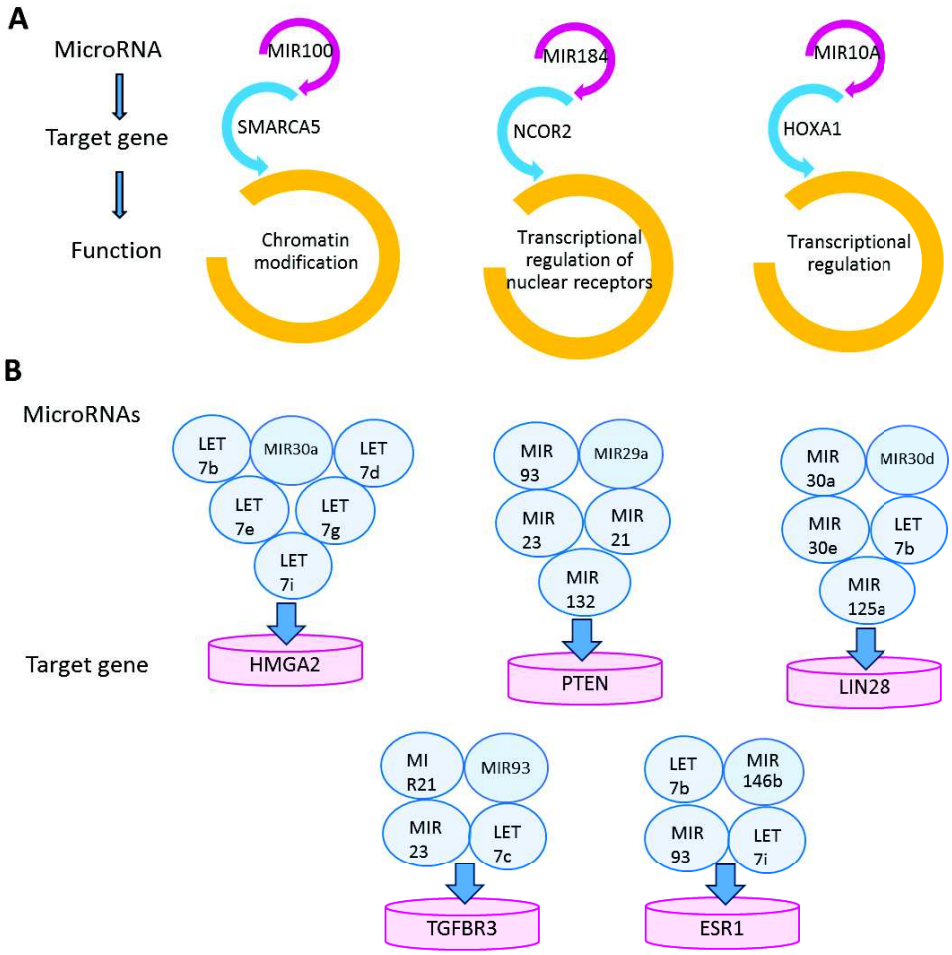

Figure 17 : Cibles des miARN identifiés dans l'ovocyte MII et les CC. (A) Les miARN identifiés dans l'ovocyte MII, ciblent essentiellement des gènes impliqués dans des fonctions nucléaires (chromatine, transcription). **(B)** Les miARN identifiés dans les CC ciblent des gènes impliqués dans l'ensemble des fonctions cellulaires (organisation de la cellule, développement, mort cellulaire, survie etc…).

1.1.2- Cibles des miARN enrichis dans le cumulus

538 ARNm cibles ont été validées expérimentalement pour les 32 miARN enrichis dans les cellules de cumulus humain. Certains des miARN ont un nombre de cibles potentielles particulièrement élevé, c'est le cas de *MIR21* (n = 115), *MIR29a* (n = 54) et *MIR23* (n = 30). Par ailleurs 12% des cibles prédites sont régulées par plus d'un miARN. le gène *HMGA2* (High Mobility Group AT-Hook 2) est ciblé par 6 miARN (*LET7b/7d/7e/7g/7i* et *MIR30a*), le gène *PTEN* (phosphatase and tensin homolog) est une cible potentielle de *MIR93*, *MIR29a*, *MIR23*, *MIR21* et *MIR132*, le gène *LIN28* a 5 régulateurs potentiels (*MIR30a*, *MIR30d*,

MIR30e, *MIR125a* et *LET7b*) quant à *TGFBR3* (TGF-beta receptor type III) et *ESR1* (estrogen receptor 1), ils sont des cibles potentielles pour 4 miARN régulateurs, d'une part *MIR21*, *MIR93*, *MIR23* et *LET7c* pour *TGFBR3* et d'autre part *MIR93*, *MIR146b*, *LET7i* et *LET7b* pour *ESR1* (Figure 17B).

1.2- Les gènes exprimés différentiellement dans l'ovocyte et les CC sont des cibles potentielles de certains des miARN identifiés

Afin d'essayer d'établir un lien entre les miR et leurs cibles potentielles en relation avec le dialogue entre l'ovocyte et son environnement somatique, nous avons établi et comparé les profils d'expression des ovocytes MII et des cumulus. L'analyse sur puce a été réalisée sur dix pools d'ovocytes MII et dix cumulus individuels. L'utilisation du logiciel SAM (avec les critères suivants : fold change ≥2 et FDR < 1%), permet de mettre en évidence 10169 gènes exprimés différentiellement entre les deux groupes. 4207 gènes sont sur-exprimés dans l'ovocyte et 5962 dans le cumulus par rapport à l'ovocyte. Ces listes de gènes ont été croisées à la liste des cibles potentielles des miR identifiés plus haut. Une liste de 224 gènes est établie sur la base de leur expression différentielle et de leur potentielle régulation par des miR identifiés par séquençage (Figure 18A). Parmi les gènes surexprimés dans l'ovocyte, on compte *CDC25A* (cell division cycle 25 homolog A), un régulateur-clé de la méiose ovocytaire (fold: 53; FDR = 0, cible de *MIR21*, *MIR424* et *LET7b*) ainsi que des gènes impliqués dans le remodelage de la chromatine tels que l'ADN methyltransferase *DNMT3B* (fold: 58; FDR = 0, cible de *MIR29a*), *DNMT1* (fold: 36; FDR = 0, cible de *MIR21*), *DNMT3A* (fold: 6; FDR = 0, cible de *MIR29a*) et *SMARCA5* (fold: 5; FDR = 0, cible de *MIR100*). L'expression de certains gènes spécifiquement exprimés dans les CC, connus pour être régulés par le facteur paracrine GDF9 (Growth differentiation factor 9), tels *PTGS2* (fold: 93; FDR = 0) et *CTGF* (fold: 38; FDR = 0), sont des cibles prédites de *MIR542* et *MIR21*, respectivement, tandis que *BMPR1B* (fold: 18; FDR = 0) est une cible prédite de *MIR21* (Figure 18B). La figure 18 schématise l'ensemble de ces données.

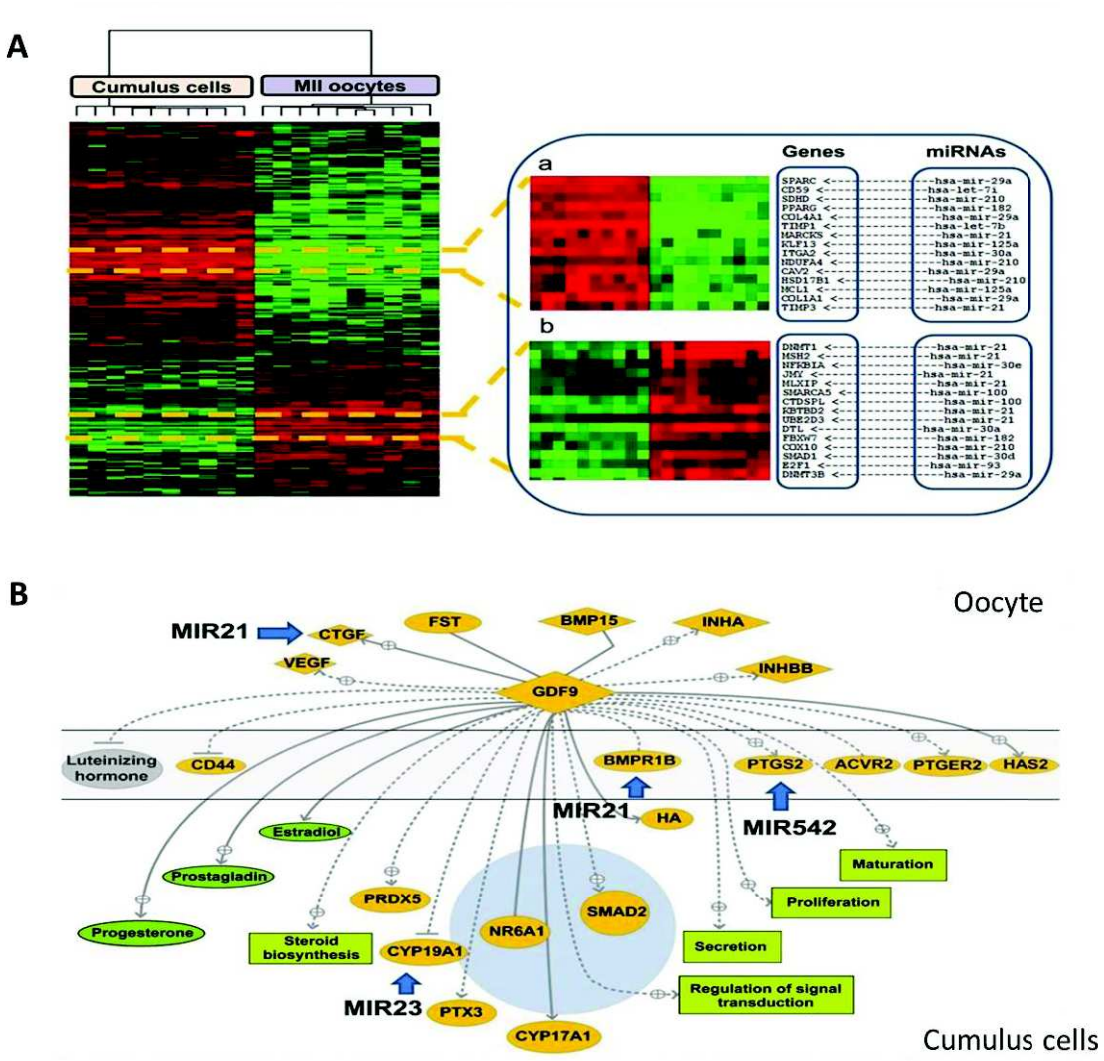

Figure 18 : Expression différentielle et miARN régulateurs. (A) Heatmap représentant les 224 gènes exprimés de manière différentielle dans les ovocytes et les CC et qui sont des cibles potentielles des miARN identifiés par séquençage profond. **(a)** Cluster de gènes cibles surexprimés dans les CC. **(b)** Cluster de gènes cibles surexprimés dans l'ovocyte MII par rapport aux CC. Code couleur : surexpression (rouge) et sous-expression (vert). **(B)** Représentation schématique des membres de la voie de signalisation de GDF9 et leurs miARN régulateurs. Les gènes *PTGS2* et *CTGF*, exprimés spécifiquement dans les CC et connus pour être régulés par le facteur GDF9 secrété par l'ovocyte, sont des cibles potentielles des miARN *MIR542* et *MIR21*, respectivement.

En conclusion, cet article constitue une première étape dans une analyse des bases moléculaires du dialogue ovocyte-cumulus au sein du follicule.

B- Résumé des travaux présentés dans l'article 2

Dans ce deuxième article, nous avons analysé l'impact de l'âge maternel sur l'expression des gènes et leurs régulateurs. Ces travaux sont fondés sur l'hypothèse que l'âge maternel pourrait impacter largement l'expression des gènes et par là-même des voies de signalisation qui sont critiques pour la qualité de l'ovocyte et le développement embryonnaire. Cette étude schématisée sur la Figure 19 présente trois points : (i) une évaluation de l'impact de l'âge des patientes sur les profils d'expression des gènes dans les cellules de cumulus, (ii) une caractérisation des voies de signalisation affectées par l'âge de façon significative et enfin (iii) une identification des miARN régulateurs.

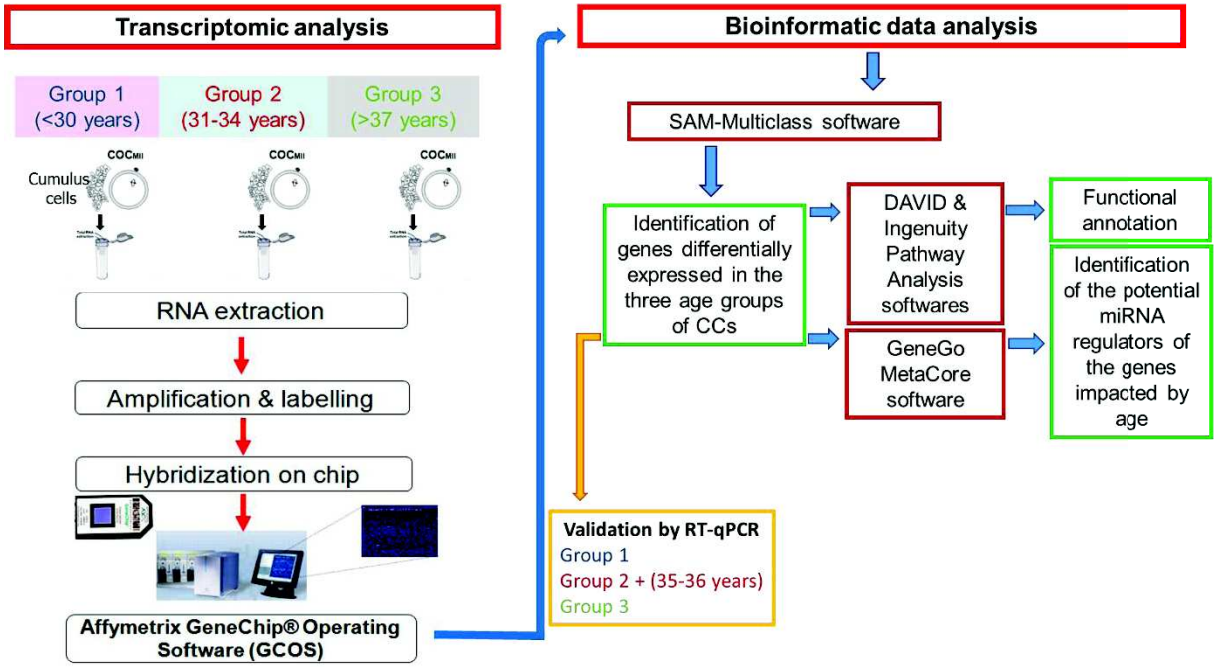

Figure 19 : Représentation schématique du protocole d'analyse des transcriptomes de cumulus en fonction de l'âge maternel. A gauche, analyse transcriptomique réalisée sur des ARN de cumulus individuels provenant de patientes de 3 classes d'âge afin d'identifier les gènes différentiellement exprimés. Au centre schéma de validation par RT-qPCR. A droite, schéma de l'analyse bioinformatique des données de transcriptome pour l'annotation fonctionnelle et l'identification des miRNA régulateurs potentiels des gènes impactés par l'âge. COC_{MII} : complexe ovocyte cumulus en métaphase 2. CC : cellules de cumulus.

1-Profils d'expression des gènes dans les cellules de cumulus en fonction de l'âge maternel

Afin de comprendre les bases moléculaires de l'impact de l'âge maternel sur la qualité des follicules ovariens, des transcriptomes ont été établis à partir de cumulus de patientes de trois catégories d'âge. L'expérience a été réalisée sur des puces Affymetrix à partir d'ARN extraits de cumulus individuels provenant de patientes de 3 catégories d'âge <30, 31-34, >37, dénommées CCyounger, CCmedian et CColder respectivement. Un premier filtre sur

l'ensemble des données de transcriptome met en évidence 9802 transcrits soumis au logiciel SAM-M. Les 35 gènes identifiés dans des travaux précédents comme étant impactés par le protocole de stimulation (Assou et al., 2013b) ont été écartés de cette analyse. Après analyse, 2,186 transcrits (correspondant à 1,874 gènes) permettent de discriminer de façon significative les CC en fonction de la classe d'âge (q-value <5%). Les profils d'expression de certains des gènes représentatifs de ces trois catégories sont illustrés par les box-plots (Figure 20A). Dans le groupe jeune (CC-younger), on observe une surexpression pour les gènes de réponses à l'inflammation tels *B4GALT1*, *SERPINA1*, *C1S*, *IL18R1*, *FN1*, et *OSMR*. Dans le groupe intermédiaire (CC-median) on observe une surexpression de gènes impliqués dans la voie de signalisation de l'insuline dont les plus représentatifs sont *IGFBP3*, *IGFBP5* et *PIK3R1*.

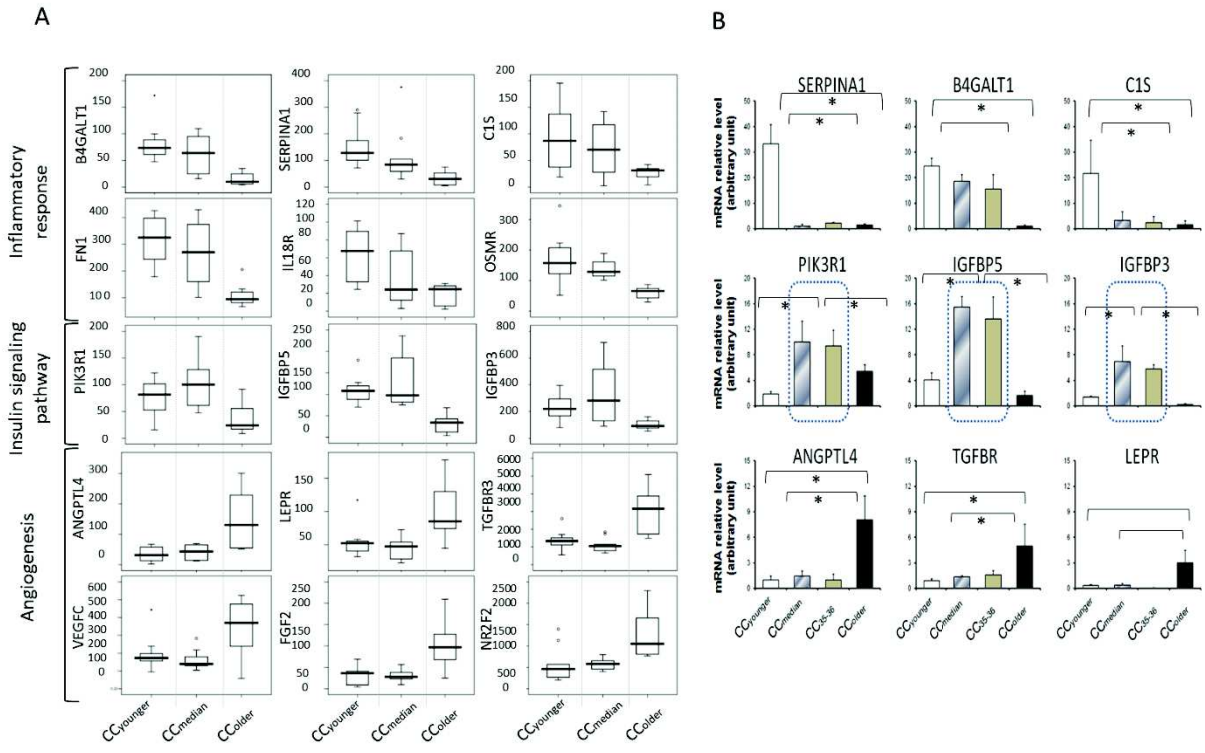

Figure 20 : Expression des gènes dans les cellules de cumulus en fonction de l'âge maternel. (A) Box plots qui représentent l'expression de gènes représentatifs des trois classes d'âge analysées par microarrays (CCyounger, CCmedian, et CColder). L'intensité du signal de chaque gène, est représentée sur l'axe *y* en unités arbitraires déterminées par le logiciel GCOS Affymetrix. **(B)** Validation par RT-qPCR de certains des gènes dont l'expression est différentielle selon l'analyse microarrays et caractéristiques de chacune des 3 classes d'âge. Trois gènes impliqués dans la réponse inflammatoire (*SERPINA1*, *B4GALT1* et *C1S*), trois dans la signalisation de l'insuline (*PIK3R, IGFBP3* et *IGFBP5*), et trois gènes dans le processus de l'angiogenèse (*ANGPTL4, TGFBR3* et *LEPR*) ont été analysés. Les histogrammes montrent l'abondance relative de l'ARNm en unités arbitraires. Blanc : CCyounger (<30 ans), gris : CCmedian (31-34 ans), noir : CColder (>37 ans). Notons qu'une catégorie supplémentaire a été ajoutée (marron : 35-36 ans). Les résultats sont présentés sous forme de moyenne ± SEM. * $p < 0,05$.

Enfin, la catégorie la plus âgée (CC-older) est enrichie de façon significative en gènes importants dans le processus d'angiogenèse tels *ANGPTL4*, *LEPR*, *TGFBR3*, *VEGFC*, *FGF2* et *NR2F2*. Toutes ces données ont été validées par RT-qPCR (Figure 20B).

Il est intéressant de souligner que la rupture se fait vraiment à 37 ans car l'analyse par RT-qPCR de l'expression des gènes sélectionnés, sur des cumulus de patientes âgées de 35-36 ans, est clairement à rapprocher de la classe CC-median. Nous avons ensuite procédé à un clustering hiérarchique sur les 20 gènes qui présentent la plus forte expression et la plus faible valeur *q* dans chaque catégorie d'âge (Figure 21).

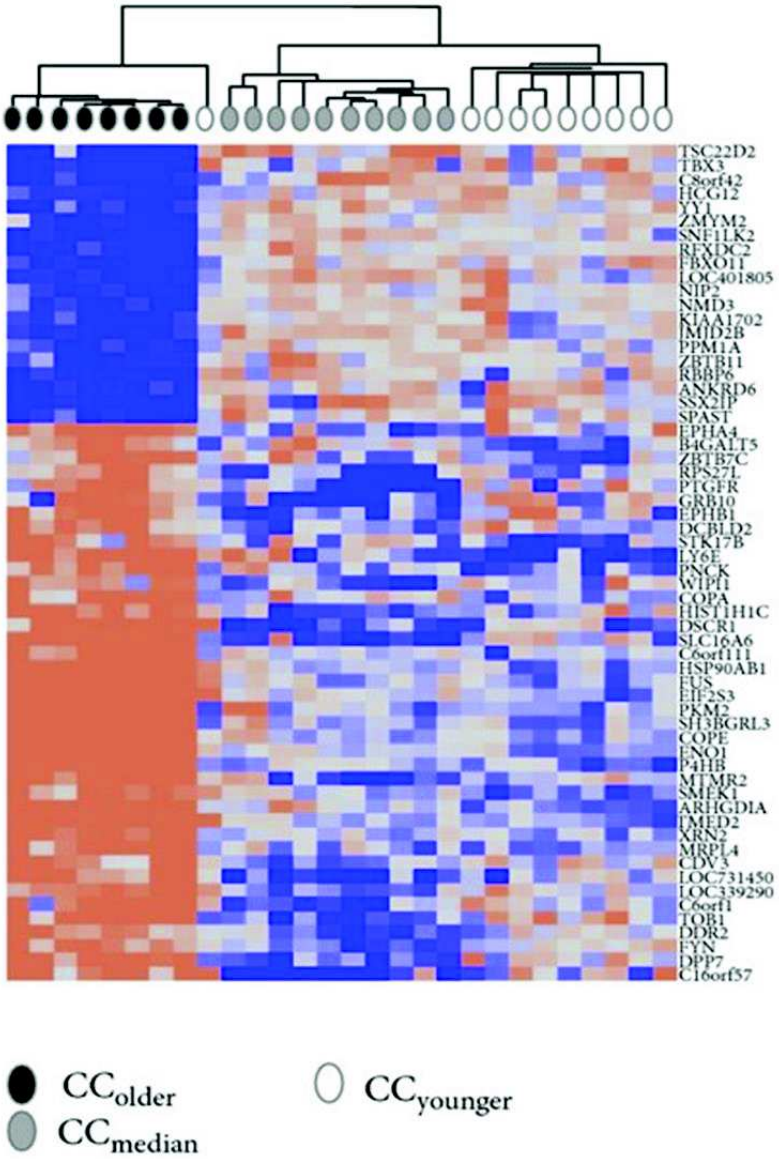

Figure 21 : Clustering hiérarchique des 20 gènes les plus impactés par l'âge. Les gènes surexprimés sont indiqués en bleu et ceux sous-exprimés, en rose. Les trois catégories d'âge sont représentées en blanc pour CCyounger, gris pour CCmedian, et noir pour CColder. Cette analyse permet de discriminer clairement la classe CColder des deux autres catégories d'âge.

Il est intéressant d'observer qu'une signature très caractéristique permet de discriminer les CC-older des autres catégories d'âge. Ces travaux permettent de conclure à un large impact de l'âge maternel sur l'expression des gènes dans le follicule et à l'existence d'une frontière moléculaire qu'on peut situer à 37 ans.

2-Identification des potentiels miARN régulateurs des gènes, dont l'expression est affectée par l'âge

Les gènes différentiellement exprimés dans les CC des trois classes d'âge ont été soumis à une recherche de leurs miARN régulateurs à l'aide du logiciel GenGo Metacore. De tous les miARN identifiés par GenGo, n'ont été retenus que les 286 dont la validation expérimentale a été rapportée dans la littérature. 176 d'entre eux sont communs aux trois classes d'âge dont 22 avaient été identifiés expérimentalement dans la première partie de nos travaux (Article 1). Sur les 110 autres miARN, 33 sont spécifiques à la classe CC-older alors que les classes CC-younger et CC-median partagent 71 miR. Par contraste un seul miR est commun à CC-older et CC-younger d'une part et CC-median d'autre part. Cette analyse permet d'illustrer de façon différente de l'analyse transcriptomique la singularité de la classe CC-older des cumulus de patientes âgées de 37 ans et plus.

Soulignons que 28 sur les 32 miARN identifiés dans l'expérience de RNAseq, sont identifiés par GenGo, à savoir 87%. N'ont été soumis à GenGo que les gènes différentiellement exprimés ce qui explique que les miARN séquencés n'aient pas été identifiés par GenGo dans leur intégralité. En revanche ces 28 miARN ne représentent qu'une petite fraction des 286 miARN identifiés par GenGo. Il est vraisemblable que notre identification expérimentale des miR ne soit pas exhaustive comme il est possible qu'une proportion non négligeable de ces régulateurs putatifs ne soit pas exprimée dans les cellules de cumulus. 6 des 28 miARN sont spécifiques : 4 pour le super-groupe CC-younger/CC-median (*MIR425, MIR744, MIR146b, LET7d*) et 2 pour le groupe CC-older (*MIR202, LET7e*) (Figure 22). Il est intéressant de souligner que *MIR202* est un régulateur potentiel de *HAS2* (hyaluronan synthase-encoding) impliqué dans le vieillissement et l'angiogenèse (Sprenger et al., 2010) et *MIR744*, un régulateur de *TGFB1* (Martin et al., 2011).

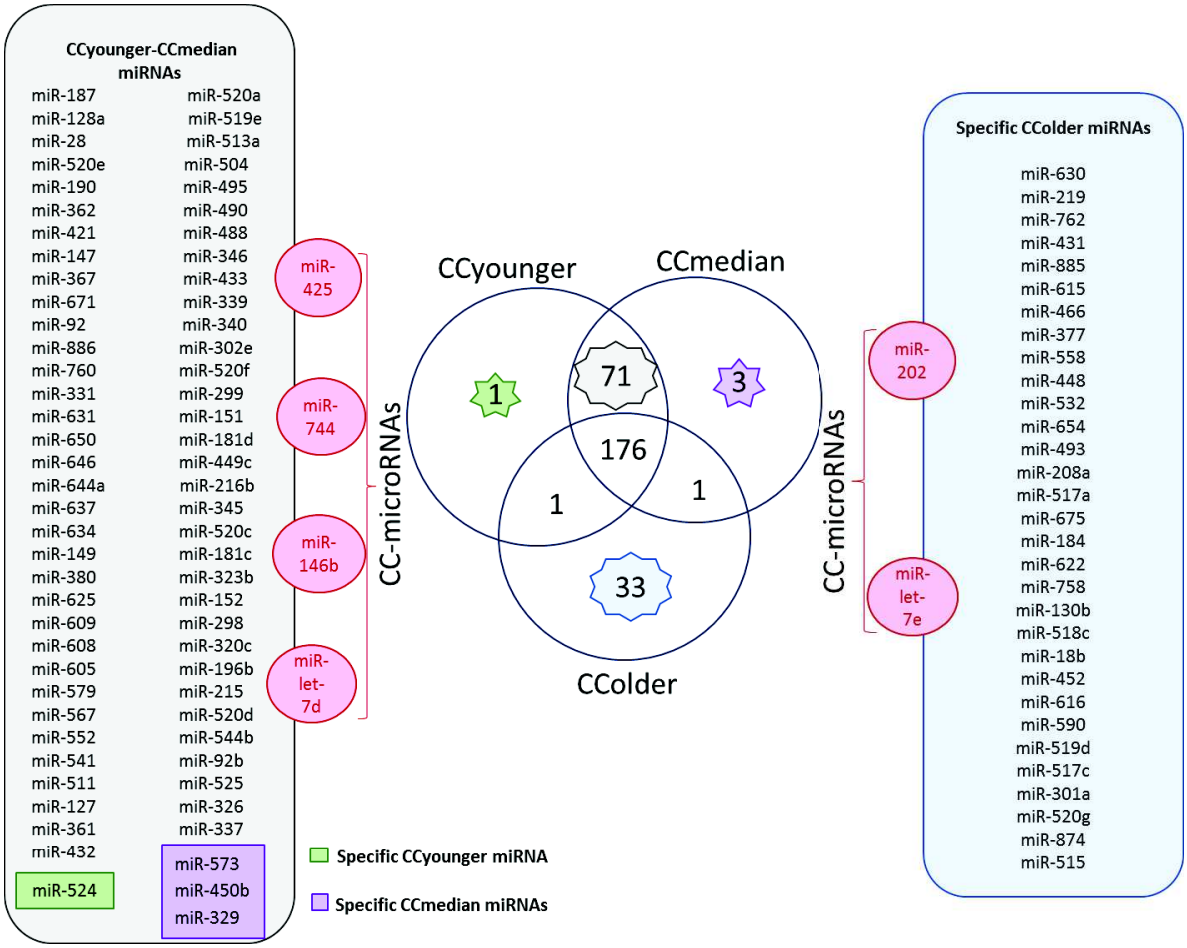

Figure 22 : Diagramme de Venn issu de l'analyse GenGo des miARN putatifs. Les gènes surexprimés dans chaque catégorie d'âge ont été soumis au logiciel GenGo pour identifier leurs potentiels miARN régulateurs. 249 miARN ont été identifiés pour le groupe de CCyounger, 251 pour le CCmedian, et 211 pour le CColder. Le diagramme de Venn permet de montrer que la majorité est commune aux trois catégories d'âge et que les catégories CCyounger/CCmedian ont un grand nombre de miARN en commun. Les miARN précédemment identifiés par séquençage profond sont indiqués en rose.

En conclusion, l'âge maternel a un large impact sur l'expression des gènes dans les cellules de cumulus. Cette analyse met en évidence sans ambiguïtés un changement moléculaire à partir de 37 ans. L'analyse bioinformatique des microARN régulateurs illustre parfaitement ce changement moléculaire lorsque l'âge maternel est de 37 ans et plus. Une analyse expérimentale de l'impact de l'âge maternel sur l'expression des miARN permettra de proposer un tableau complet de l'impact de l'âge sur le transcriptome et ses régulateurs dans le cumulus humain.

II- Articles originaux

- Article 1

Assou S, **Al-edani T**, Haouzi D, Philippe N, Lecellier C-H, Piquemal D, Commes T, Aït Ahmed O, Dechaud H, Hamamah S. MicroRNAs: new candidates for the regulation of the human cumulus-oocyte complex (2013). *Hum Reprod 2013; 28, 3038-49.*

Human Reproduction, Vol.28, No.11 pp. 3038–3049, 2013
Advanced Access publication on July 30, 2013 doi:10.1093/humrep/det321

human reproduction

ORIGINAL ARTICLE *Reproductive biology*

MicroRNAs: new candidates for the regulation of the human cumulus–oocyte complex

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Submitted on February 6, 2013; resubmitted on July 2, 2013; accepted on July 11, 2013

STUDY QUESTION: What is the expression pattern of microRNAs (miRNAs) in human cumulus–oocyte complexes (COCs)?

SUMMARY ANSWER: Several miRNAs are enriched in cumulus cells (CCs) or oocytes, and are predicted to target genes involved in biological functions of the COC.

WHAT IS KNOWN ALREADY: The transcriptional profiles of human MII oocytes and the surrounding CCs are known. However, very limited data are available about post-transcriptional regulators, such as miRNAs. This is the first study focussing on the identification and quantification of small RNAs, including miRNAs, in human oocytes and CCs using a deep-sequencing approach.

STUDY DESIGN, SIZE, DURATION: MII oocytes and CCs were collected from women who underwent IVF.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Using the Illumina/deep-sequencing technology, we analyzed the small RNAome of pooled MII oocytes ($n = 24$) and CC samples ($n = 20$). The mRNA targets of CC and MII oocyte miRNAs were identified using *in silico* prediction algorithms. Using oligonucleotide microarrays, genome-wide gene expression was studied in oocytes (10 pools of 19 $\pm$ 3 oocytes/each) and 10 individual CC samples. TaqMan miRNA assays were used to confirm the sequencing results in independent pools of MII oocytes (3 pools of 8 $\pm$ 3 oocytes/each) and CC samples (3 pools of 7 $\pm$ 3 CCs/each). The functional role of one miRNA, *MIR23a*, was assessed in primary cultures of human CCs.

MAIN RESULTS AND THE ROLE OF CHANCE: Deep sequencing of small RNAs yielded more than 1 million raw reads. By mapping reads with a single location to the human genome, known miRNAs that were abundant in MII oocytes (*MIR184*, *MIR100* and *MIR10A*) or CCs (*MIR29a*, *MIR30d*, *MIR21*, *MIR93*, *MIR320a*, *MIR125a* and the *LET7* family) were identified. Predicted target genes of the oocyte miRNAs were associated with the regulation of transcription and cell cycle, whereas genes targeted by CC miRNAs were involved in extracellular matrix and apoptosis. Comparison of the predicted miRNA target genes and mRNA microarray data resulted in a list of 224 target genes that were differentially expressed in MII oocytes and CCs, including *PTGS2*, *CTGF* and *BMPR1B* that are important for cumulus–oocyte communication. Functional analysis using primary CC cultures revealed that *BCL2* and *CYP19A1* mRNA levels were decreased upon *MIR23a* overexpression.

LIMITATIONS, REASONS FOR CAUTION: Only known miRNAs were investigated in the present study on COCs. Moreover, the source of the material is MII oocytes that failed to fertilize.

WIDER IMPLICATIONS OF THE FINDINGS: The present findings suggest that miRNA could play a role in the regulation of the oocyte and CC crosstalk.

STUDY FUNDING/COMPETING INTEREST(S): This work was partially supported by a grant from Ferring Pharmaceuticals. The authors of the study have no conflict of interest to report.

TRIAL REGISTRATION NUMBER: Not applicable.

Key words: MicroRNAs / oocyte / cumulus cells / deep sequencing / microarray

Introduction

The quality of oocytes obtained during IVF procedures varies considerably. Whilst most mature oocytes are amenable to fertilization, only half of those fertilized complete embryonic development and fewer implant. In the ovarian follicle, the maturing oocyte is nurtured and supported by cumulus cells (CCs), the surrounding somatic cells. CCs are highly specialized cells with trans-zonal cytoplasmic projections that form gap junctions at the oocyte surface (Albertini *et al.*, 2001) as part of the cumulus–oocyte complex (COC) (Cha and Chian, 1998; Goud *et al.*, 1998; Barrett and Albertini, 2010). Disruption or deregulation of the CC interactions with the oocyte can affect oocyte quality and consequently embryo development and pregnancy outcome. Much knowledge on human oocytes and CCs has been generated over recent years mainly owing to technological advances in gene expression analysis using microarray (Assou *et al.*, 2006; Gasca *et al.*, 2007; Assou *et al.*, 2009; Assou *et al.*, 2011), CGH array (Gutierrez-Mateo *et al.*, 2004; Fragouli *et al.*, 2010) and high-fidelity RNA amplification (Wood *et al.*, 2007). Such techniques have also allowed entire profiling of the transcriptional activity in single human oocytes (Grondahl *et al.*, 2010). We and others have identified several transcripts in human MII oocytes and the surrounding CCs that are crucial for oogenesis and folliculogenesis (Assou *et al.*, 2006; Kocabas *et al.*, 2006). However, the post-transcriptional regulation of oocyte and CC transcripts needs to be elucidated. This is particularly important also because the stability and translation of the maternal mRNAs, that are accumulated during oocyte maturation (Niakan *et al.*, 2012) and that drive human preimplantation development, are controlled by post-transcriptional regulatory mechanisms (Bettegowda and Smith, 2007).

Recently, it has been demonstrated that small (~19–25 nucleotides in length) endogenous non-coding transcripts, called microRNAs (miRNAs), execute key functions by silencing the expression of specific target genes in plant, animal and human genomes (Reinhart *et al.*, 2002; Lewis *et al.*, 2005; Nilsen, 2007; Krol *et al.*, 2010). In addition, miRNAs are involved in the regulation of many cellular processes, including cell proliferation, differentiation and apoptosis (Bartel, 2004). The miRNA repertoires are cell type specific and change markedly during development (Carthew and Sontheimer, 2009). Changes in miRNA expression profiles have been linked to pathologies, such as cancer (Ventura and Jacks, 2009). Moreover, miRNAs have been associated with infertility as shown in female mice in which Dicer, an essential factor in miRNA biogenesis, was genetically ablated (Murchison *et al.*, 2007; Nagaraja *et al.*, 2008). Futhermore, analysis of messenger RNA (mRNA) expression during mouse and bovine oogenesis shows that a large proportion of maternal genes are regulated by miRNAs (Tang *et al.*, 2007; Lingenfelter *et al.*, 2011). Thus, miRNA profiling might help us to better understand the regulation of transcripts involved in human reproduction.

The aim of the present study was (i) to identify and quantify small RNAs, including miRNAs, in human CCs and MII oocytes and (ii) to characterize the biological relationships between miRNAs and the mRNA expression profiles of MII oocytes and CCs.

Materials and Methods

Sample collection and processing

Human MII oocytes that failed to fertilize and CCs were collected from patients who underwent conventional IVF or ICSI. All patients signed informed consent forms. Moreover, the material used in the present study would have been discarded as all the MII oocytes used were IVF by-products.

Oocytes and CCs

MII oocytes that failed to fertilize were collected 24 h post-insemination as previously described (Assou *et al.*, 2006; Monzo *et al.*, 2012) and CCs were mechanically removed from MII oocytes before ICSI. MII oocytes were pooled for sequencing, microarray and validation by RT–qPCR. CCs were pooled for sequencing and RT–qPCR validation, whereas 10 individual CCs were used for microarray analysis (Supplementary data, Table SI). All samples were immediately transferred in 0.5 ml Eppendorf® tubes containing RLT lysis buffer (ref: 74004; Qiagen) and frozen at −80°C.

RNA extraction

The RNeasy Micro Kit (ref: 74004; Qiagen) was used to isolate both total and small RNAs from MII oocytes and CCs. Small RNA was extracted as described in the manufacturer's protocol for the RNeasy Micro Kit (ref: 74004; Qiagen) with the following modifications: the lysate/RLT was equilibrated at 37°C for a few minutes prior to RNA purification, and after addition of the carrier RNA and lysis (0.2 ng/μl), 1.5 volumes of 100% ethanol were added to the lysates instead of one volume of 70% ethanol. Total RNA (5 ng/μl for oocytes and 2 μg/μl for CCs) was quantified using a NanoDrop® ND-1000 spectrophotometer (NanoDrop ND-Thermo Fisher Scientific, Wilmington, DE, USA) and its integrity assessed by using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA, http://www.agilent.com).

Preparation of the small RNA cDNA libraries and sequencing

Small RNA cDNA libraries were prepared according to the Illumina's V1.0 protocol. The 5′ RNA adaptor (5′-GUUCAGAGUUCUACAGUCCGA CGAUC-3′) was ligated to the oocyte and CC small RNA pools (Supplementary data, Table SI) with 1 μl of T4 RNA ligase (10 U/μl) (ref: M0242L; NEB) in the presence of RNase Out (ref: 10777-019; Invitrogen) overnight at 25°C. The ligation reaction was stopped by addition of 2× formamide loading dye and size fractionated on a 15% TBE urea polyacrylamide gel. The 40–60 base pair fraction (RNA plus 5′ adaptor) was excised and the RNA was eluted by incubating the gel slice at 4°C overnight in 600 μl NaCl 0.3 M, precipitated and suspended in DEPC-treated water. The 3′ RNA adapter (5′-pUC GUAUGCCGUCUUCUGCUUGidT-3′; p, phosphate; idT, inverted deoxythymidine) was then ligated to the RNA at 25°C overnight with T4 RNA ligase (NEB) in the presence of RNase Out (Invitrogen). The RNA with the 5′ and 3′ adaptors was size fractionated on a 10% TBE urea polyacrylamide and the 60–100 base pair RNA fraction was extracted as described above. Superscript II reverse transcriptase (Invitrogen) was used to reverse transcribe the RNA using the Illumina small RNA RT-Primer (5′-CAAGCA GAAGACGGCATACGA-3′). The resulting cDNA was submitted to 15 amplification cycles using Hotstart Phusion DNA Polymerase (NEB) and the Illumina small RNA primer set (5′-CAAGCAGAAGACGGCAT ACGA-3′; 5′-AATGATACGGCGACCACCGA-3′). After purification on a 12% TBE urea polyacrylamide gel, the PCR products were eluted in buffer (5:1, 7.5 M ammonium acetate) at 4°C overnight. The resulting gel slurries were submitted to Spin-X filters (Corning) to purify the PCR products before ethanol precipitation and pellet suspension in water. The DNA was quantified using an Agilent DNA 1000 chip and diluted to 10 nM for sequencing using an Illumina 1G sequencer.

Complementary RNA preparation and microarray processing

Total RNA samples from 10 pools of MII oocytes (2.7 ± 1.4 ng/μl per pool) (Supplementary data, Table SI) were subjected to two rounds of linear amplification according to the manufacturer's 'double amplification' protocol (two-Cycle cDNA Synthesis Kit; Invitrogen). Labeled fragmented cRNA was hybridized to HG-U133 plus 2.0 GeneChip arrays (Affymetrix, Santa Clara, CA, USA) as described in Assou *et al.* (2006, 2009; Monzo *et al.* 2012). Total RNA (50 ng) from 10 individual CCs was used to prepare cRNA using the Affymetrix 3′ IVT express protocol (ref.901229) as described in (Ouandaogo *et al.*, 2011). After fragmentation, the labeled anti-sense aRNA (15 μg) was hybridized to HG-U133 plus 2.0 GeneChip arrays (Affymetrix™). Each CC sample was processed individually on a microarray chip.

Data processing and gene expression profile analysis

After image processing using the Affymetrix Microarray Suite 5.0, the .CEL files were analyzed using the Affymetrix Expression Console™ software and normalized with the MAS5.0 algorithm by scaling each array to a target value of 100 using the global scaling method to obtain an intensity value signal for each probe set. Gene annotation was performed using NetAffx (http://www.affymetrix.com; March 2009). Genes with significant differential expression profiles between MII oocytes and CC samples were identified using the significance analysis of microarray (SAM) algorithm (http://www-stat.stanford.edu/~tibs/SAM/), which utilizes a Wilcoxon test statistic and sample-lable permutation to evaluate statistical significance between sample groups. SAM provides mean fold change values (FC $>$ 2) and a false discovery rate (FDR $<$ 5%) confidence percentage based on data permutation ($n = 300$). Hierarchical clustering was carried out with CLUSTER and TREEVIEW software (Eisen *et al.*, 1998).

Small RNA annotation and deep-sequencing data analysis pipeline

First, short sequences of 19–22 nucleotides (nt) in length were independently analyzed as described in Philippe *et al.* (2009). Briefly, reads were mapped with the CRAC software and reads with a single location were annotated with a double-step process according to the ENSEMBL Genome Browser (Ensembl API version 66, http://www.ensembl.org/index.html): the distribution of reads relative to protein coding genes (exonic, intronic or intergenic part) and the distribution in non-coding regions (Fig. 1A). Then, all the annotated non-coding transcripts were compiled to specify their frequency and distribution. Finally, the GeneGo MetaCore pathway analysis software (St. Joseph, MI), which provides predicted validated targets for known miRNAs, was used for miRNA target prediction.

Taqman miRNA assays

Complementary DNA was synthesized from total RNA from pooled MII oocytes or CCs (Supplementary data, Table SI) using the TaqMan miRNA-specific primers, *LET7b*, *MIR21*, *MIR30d*, *MIR184* and *MIR10A* (ref: #4427975, Life Technologies), according to the TaqMan MicroRNA RT protocol (Applied Biosystems). For reverse transcription, 5 μl (10 ng) of RNA sample, 0.15 μl (100 mM) dNTPs, 1 μl of 50 U μl^{-1} MultiScribe reverse transcriptase, 1.5 μl 10× RT buffer, 0.19 μl of 20 U μl^{-1} RNase inhibitor and 3 μL of 50 nM stem-loop RT primer (all from the TaqMan MicroRNA Reverse Transcription Kit; Applied Biosystems) were used. Reaction mixtures (15 μl) were incubated first at 16°C for 30 min and then at 42°C for 30 min, inactivated at 85°C for 5 min and then stored at 4°C. Quantitative PCR was performed using a Roche LightCycler 480 apparatus. The 10 μl PCR reaction mixtures included 4 μl of RT product, 4.5 μl 2× TaqMan (AmpErase UNG) Universal PCR Master Mix and 0.5 μl of primer and probe mix from the TaqMan MicroRNA Assay kit (ref: 4324018; Applied Biosystems). Reaction mixtures were incubated in a 384-well plate at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and at 60°C for 60 s. *RN U6-1* was used as reference gene for normalization of the miRNA expression levels. This endogenous gene control showed a stable expression pattern between CCs and oocyte samples. The relative expression levels of target miRNAs were determined by using the equation $2^{-\Delta CT}$, in which ΔC_T were calculated as follows:

$$\Delta C_T = C_{T\,\text{miRNA of interest}} - C_{T\,\text{RN U6-1}}$$

CC culture and miRNA transfection

The fresh CCs were mechanically separated from the oocyte by using two needles. One needle was placed on the CC layer to keep the oocyte in place. The other needle was used to quickly cut off as much as possible of the cell layer, without damaging the oocyte. The CC clumps were transferred into a dish coated with 10 $\mu g/cm^2$ type I–III human collagen (in alpha-MEM medium) and cultured in serum-free medium (SPE-IV/EBM). At confluence, cells were washed by PBS and detached with TrypLE™ Select (3436D; Life Technologies) treatment for 5 min at 37°C. They were then seeded onto new culture dishes treated by human collagen I–III for expansion. The following experiments were performed on a primary culture of CCs obtained from one patient at passage 3 (P3). These cells were cultured in 100 mm culture dishes with an estimated plating density of 2.5×10^5 cells/well. When cells reach 50–60% confluence after 2 days in culture, they were transfected with 5 μg of wild-type *MIR23a* locus or *MIRD23* (mutated *MIR23a* locus that expresses only *MIR24* and *MIR27a*) cloned into the MIE retroviral vector using jetPEI® (www.polyplus-transfection.com) (Rathore *et al.*, 2012). The miR-23 constructs were tagged with GFP in order to assess the transfection efficiency, which ranges between 70 and 80%. Total RNA was extracted 48 h after transfection to perform quantitative RT–PCR analysis. The transfection experiments were repeated three times on P3 CC cultures and the qRT–PCR experiments were performed in triplicate. We verified that the P3 cells used in the above experiment retained the properties of CCs at the first passage (P1) by RT–qPCR analysis of three genes known to be linked to CC function, namely *AREG*, *STAR* and *PTX3*. It was reported in granulosa cell (GC) primary cultures that the cells retained their properties up to four passages (Brůcková *et al.*, 2008).

Quantitative PCR for mRNA

Total RNA derived from the primary CC cultures was reverse transcribed in a final volume of 20 μl with the SuperScript® First-Strand Synthesis System (Invitrogen) according to the manufacturer's instructions. Quantitative PCR was performed in 384-well plates (Sorenson BioScience, Inc.) on a Lightcycler® 480 Real-Time PCR System (Roche Diagnostics) using a reaction mix (final volume of 10 μl) that contained 2 μl cDNA, 5 μl SybrGreen (Roche Diagnostics) and 0.5 μM forward and reverse primers (*BAX*, forward primer 5′-CCAGCTGCCTTGGACTGT-3′ and reverse primer 5′-ACCC CCTCAAGACCACTCTT-3′; *BCL2*, forward primer 5′-GGCTGATATT CTGCAACACTG-3′ and reverse primer 5′-GGCAATGTGACTTTTT CCAA-3′; *CYP19A1*, forward primer 5′-TGCAAAGCACCCTAATGTTG-3′ and reverse primer 5′-TTTGTCCCCTTTTTCACTGG-3′) and the following conditions: incubation at 95°C for 10 min, then 40 cycles of 10 s at 95°C, 20 s at 63°C and 25 s at 72°C. At the end, a melting curve from 95 to 63°C was performed to control primer specificity. The geometric mean of the *GAPDH* showed stable expression pattern across the treatment groups that were compared. Therefore, *GAPDH* was used as endogenous control.

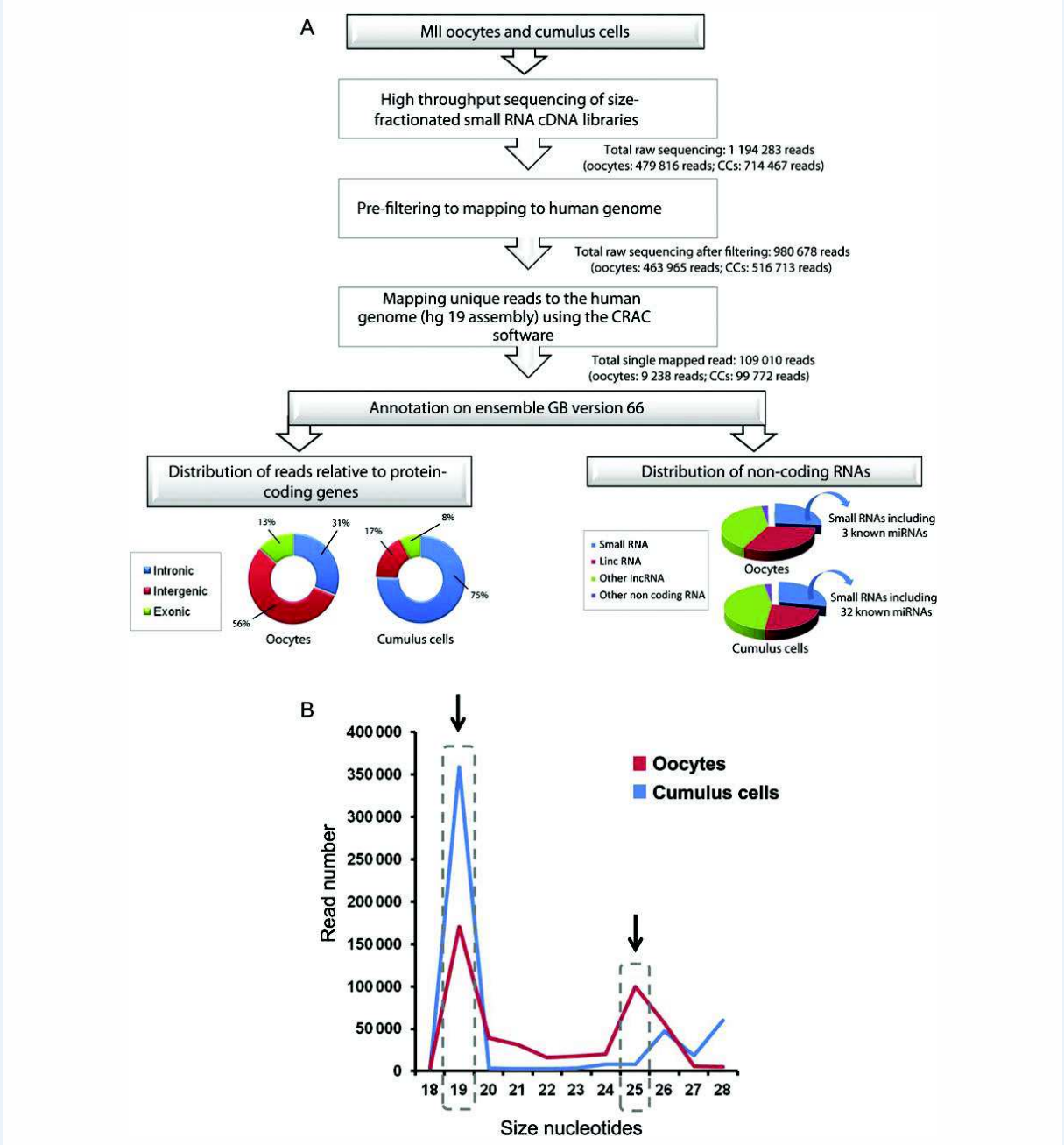

Figure 1 Computational pipeline for the analysis of the deep-sequencing data. (**A**) Small RNA cDNA libraries from human MII oocytes, and CCs were subjected to deep sequencing. Raw sequence reads were filtered, mapped to the reference human genome using the CRAC software and annotated according to the ENSEMBL Genome Browser (Ensembl API version 66). The location (intergenic, intronic or exonic) of reads that perfectly mapped to a single location in the human genome was determined. The distribution of the non-coding RNA classes, including small RNA, large intergenic non-coding RNAs (lincRNAs), other large non-coding RNAs (lncRNAs) and other small RNAs in the MII oocyte or CC genomes is given as a percentage of all mapped tags. The small RNA category (blue) contains known miRNAs. (**B**) Size distribution of the sequence reads. The two histograms show the length distribution of small RNAs in the MII oocyte and CC libraries.

Statistical analysis

The quantitative PCR results were expressed as the mean ± standard error of mean. One-way ANOVA was used for multiple comparisons, using SPSS for Windows (SPSS 16.0, SPSS Inc., Chicago, USA) and *t*-test was used for comparison between two groups; $P < 0.05$ was considered as significant.

Results

Deep sequencing of the small RNAome of human MII oocytes and CCs

Deep sequencing of the two small RNA cDNA libraries from human MII oocytes and CCs produced over 1 million separated reads. The pipeline approach used to analyze the sequences and identify known miRNAs is summarized in Fig. 1A. Most reads had an intronic (75% of CC and 31% of MII oocyte reads) or intergenic (56% of MII oocyte reads) location (Fig. 1A). In both libraries, reads showed a size distribution peak at 19 nt (Fig. 1B) that corresponded to miRNAs and represented 22% of the reads in the CC library. Reads of the MII oocyte library had also a second size distribution peak at around 25–27 nt that should correspond to Piwi-interacting RNA (pi-RNA)-like sequences (26–31 nt in size) (Fig. 1B).

miRNAs expression in human MII oocytes and CCs

Only three known miRNAs were identified in the MII oocyte library, whereas the number of known miRNAs increased to 32 in the CC library (Table I). The most abundant miRNAs in CCs were *LET7b* (51 reads), *LET7c* (31 reads) and *MIR21* (28 reads). In MII oocytes, the most abundant miRNAs were *MIR184* (1988 reads) and *MIR10A* (555 reads). To validate the sequencing data, the relative expression levels of five randomly selected miRNAs was assessed in independent pools of mature MII oocytes and CCs (Supplementary data, Table SI) by RT-quantitative PCR. The results were in accordance with the sequencing data (Fig. 2).

Identification of miRNA targets and their function

Using the GenGo Metacore software, we found that 30 mRNAs, predicted and experimentally validated by other laboratories to have roles in transcription regulation and cell cycle, were targeted by the three miRNAs identified in the MII oocyte library (*MIR184*, *MIR100* and *MIR10A*) (Supplementary data, Table SII). For instance, *SMARCA5* (*SWI/SNF*-related matrix associated actin-dependent regulator of chromatin, subfamily a member 5) was shown to be a target of *MIR100* (Bhushan and Kandpal, 2011); interestingly *SMARCA5* may be implicated in oocyte reprogramming (Assou *et al.*, 2009). *NCOR2* (nuclear receptor co-repressor 2), which mediates the transcriptional repression activity of nuclear receptors, was a target of *MIR184* (Wu *et al.*, 2011). Finally *HOXA1*, a homeobox gene whose mRNA is abundant in oocytes was targeted by *MIR10A* (Lund, 2010); it is noteworthy that *HOXA1* is essential for the regulation of oocyte-specific gene expression in the mouse (Huntriss *et al.*, 2006). The 32 miRNAs enriched in CCs (Supplementary data, Table SIII) targeted 538 mRNAs experimentally validated mRNAs by other laboratories to be involved in several biological functions, including cell assembly and organization, development, cell death and survival. The CC miRNAs with the highest number of predicted mRNA targets were *MIR21* ($n = 115$), *MIR29a* ($n = 54$) and *MIR23* ($n = 30$). Moreover, more than 12% of the predicted mRNA targets were regulated by more than one miRNA. For instance, *HMGA2* (high mobility group AT-hook 2) was targeted by six CC miRNAs (*LET7b/7d/7e/7g/7i* and *MIR30a*), *PTEN* (phosphatase and tensin homolog) by five (*MIR93*, *MIR29a*, *MIR23*, *MIR21* and *MIR132*), *LIN28* by five (*MIR30a*, *MIR30d*, *MIR30e*, *MIR125a* and *LET7b*), *TGFBR3* (TGF-beta receptor type III) by four (*MIR21*, *MIR93*, *MIR23* and *LET7c*) and *ESR1* (estrogen receptor 1) also by four (*MIR93*, *MIR146b*, *LET7i* and *LET7b*) (Supplementary data, Table SIII). As a preliminary experiment to assess the effect of *MIR23a* on a predicted target, we analyzed the impact of its forced expression on *BCL2* mRNA level in primary cultures of human CCs isolated from mature COCs. *BCL2* mRNA level decreased upon *MIR23a* overepression as did the *CYP19A1* mRNA. In contrast, BAX mRNA that is not predicted as a target did not display a decrease but rather a significant increase (Fig. 3). It is worth mentioning that *BAX* is a pro-apoptotic gene, whereas *BCL2* is anti-apoptotic. Further experiments are required to propose a mechanism to account for these results.

Genes that are differentially expressed in oocyte and CCs are predicted targets of oocyte and CC miRNAs

To explore the biological relationships between miRNAs and the mRNA expression profiles of MII oocytes and CCs, microarray analyses were performed using 10 pools of MII oocytes or 10 individual CCs (Supplementary data, Table SI). Using SAM (with a fold change ≥2 and FDR < 1%), we identified a total of 10 169 genes that were differentially expressed in the two groups. Overall, 4207 genes were specifically up-regulated in MII oocytes and 5962 genes were up-regulated in individual CCs (Supplementary data, Tables SIV and SV). Comparison of these differentially expressed mRNAs with the predicted miRNA target genes resulted in a list of 224 genes (Supplementary data, Table SVI) that included genes that were up-regulated in MII oocytes and significantly down-regulated in CCs, such as *CDC25A* (cell division cycle 25 homolog A), a key regulator of oocyte meiosis (fold: 53; FDR = 0; target of *MIR21*, *MIR424* and *LET7b*), and genes associated with chromatin remodeling, such as DNA methyltransferase *DNMT3B* (fold: 58; FDR = 0; a target of *miR-29a*), *DNMT1* (fold: 36; FDR = 0; a target of *MIR21*), *DNMT3A* (fold: 6; FDR = 0; a target of *MIR29a*) and *SMARCA5* (fold: 5; FDR = 0; a target of *MIR100*). The expression of some CC-specific genes known to be regulated by the oocyte-secreted paracrine factor *GDF9* (Growth differentiation factor 9), such as prostaglandin-endoperoxide synthase 2 *PTGS2* (fold: 93; FDR = 0) and the connective tissue growth factor *CTGF* (fold: 38; FDR = 0), were predicted targets of *MIR542* and *MIR21*, respectively, and bone morphogenetic protein receptor *BMPR1B* (fold: 18; FDR = 0) was a predicted target of *MIR21* (Fig. 4A). Additionally, *MIR21* was predicted to target many transcription factors up-regulated in CCs, such as nuclear factor *NFIB* (fold: 49; FDR = 0), myocyte enhancer factor *MEF2C* (fold: 33; FDR = 0) and retinoid X receptor alpha *RXRA* (fold: 4; FDR = 0) and *MIR29a* was predicted to target several extracellular matrix (ECM) genes up-regulated in CCs, such as collagen *COL4A1* (fold: 52; FDR = 0), *COL3A1* (fold: 6; FDR = 0), *COL1A1* (fold: 24; FDR = 0) and *COL1A2* (fold: 9; FDR = 0). Moreover, many pro-apoptotic genes that were up-regulated in CCs, including

Table I Putative miRNAs identified in human CCs and MII oocytes

Micro RNAs	Ensembl ID	Occ.	Sequence	Chr.	Location
CCs					
hsa-LET7b	ENSG00000207875	51	TGAGGTAGTAGGTTGTGTG	22	46 509 570
hsa-LET7c	ENSG00000199030	31	TGAGGTAGTAGGTTGTATG	21	17 912 157
hsa-MIR21	ENSG00000199004	28	TAGCTTATCAGACTGATGT	17	57 918 633
hsa-MIR182	ENSG00000207990	7	CAATGGTAGAACTCACACT	7	129 410 286
hsa-MIR30d	ENSG00000199153	5	TGTAAACATCCCCGACTGG	8	135 817 164
hsa-MIR99b	ENSG00000207550	4	CACCCGTAGAACCGACCTT	19	52 195 870
hsa-MIR320a	ENSG00000208037	4	GCTGGGTTGAGAGGGCGAA	8	22 102 486
hsa-MIR132	ENSG00000207724	4	TAACAGTCTACAGCCATGG	17	1 953 225
hsa-MIR191	ENSG00000207605	3	CAACGGAATCCCAAAAGCA	3	49 058 108
hsa-MIR146b	ENSG00000202569	3	TGAGAACTGAATTCCATAG	10	104 196 276
hsa-MIR93	ENSG00000207757	2	CAAAGTGCTGTTCGTGCAG	7	99 691 441
hsa-MIR744	ENSG00000211589	2	TGCGGGGCTAGGGCTAACA	17	11 985 225
hsa-MIR508	ENSG00000207589	2	TGATTGTAGCCTTTTGGAG	X	146 318 466
hsa-MIR30a	ENSG00000207827	2	TGTAAACATCCTCGACTGG	6	72 113 300
hsa-MIR23	ENSG00000207563	2	ATCACATTGCCAGGGATTA	9	97 847 546
hsa-MIR140	ENSG00000208017	2	ACCACAGGGTAGAACCACG	16	69 967 045
hsa-LET7i	ENSG00000199179	2	TGAGGTAGTAGTTTGTGCT	12	62 997 470
hsa-LET7g	ENSG00000199150	1	TGAGGTAGTAGTTTGTACA	3	52 302 354
hsa-LET7e	ENSG00000198972	1	TGAGGTAGGAGGTTGTATA	19	52 196 045
hsa-LET7d	ENSG00000199133	1	AGAGGTAGTAGGTTGCATA	9	96 941 122
hsa-MIR542	ENSG00000207784	1	TGTGACAGATTGATAACTG	X	133 675 396
hsa-MIR425	ENSG00000199032	1	TGACACGATCACTCCCGTT	3	49 057 633
hsa-MIR424	ENSG00000199097	1	CAGCAGCAATTCATGTTTT	X	133 680 712
hsa-MIR379	ENSG00000199088	1	TGGTAGACTATGGAACGTA	14	101 488 407
hsa-MIR30e	ENSG00000198974	1	TGTAAACATCCTTGACTGG	1	41 220 042
hsa-MIR29a	ENSG00000198981	1	TAGCACCATCTGAAATCGG	7	130 561 509
hsa-MIR25	ENSG00000207547	1	CATTGCACTTGTCTCGGTC	7	99 691 196
hsa-MIR210	ENSG00000199038	1	CTGTGCGTGTGACAGCGGC	11	568 114
hsa-MIR202	ENSG00000199089	1	GAGGTATAGGGCATGGGAA	10	135 061 041
hsa-MIR197	ENSG00000207709	1	CACCACCTTCTCCACCCAG	1	110 141 563
hsa-MIR183	ENSG00000207691	1	TATGGCACTGGTAGAATTC	7	129 414 809
hsa-MIR125a	ENSG00000208008	1	TCCCTGAGACCCTTTAACC	19	52 196 520
MII oocytes					
hsa-MIR184	ENSG00000207695	1988	TGGACGGAGAACTGATAAG	15	79 502 181
hsa-MIR10A	ENSG00000207777	555	TACCCTGTAGATCCGAATTTGT	17	46 657 266
hsa-MIR100	ENSG00000207994	38	AACCCGTAGATCCGAACTT	11	122 022 985

Occ, occurrence; Chr, chromosome.

BCL2-associated X protein *BAX* (fold: 14; FDR = 0), caspase *CASP7* (fold: 6; FDR = 0) and *CASP3* (fold: 2; FDR = 0), were predicted targets of the *LET7* family (*LET7b*/7d/7g/7e), *MIR30d* and *MIR29a*. Anti-apoptotic genes that were up-regulated in CCs, such as myeloid cell leukemia sequence *MCL1* (fold: 23; FDR = 0) and *BCL2* (fold: 4; FDR = 0), were predicted to be targeted by *MIR29a*, *MIR125a*, *MIR21* and *MIR30e*, whereas *BIRC5/Survivin* (fold: 7; FDR = 0), which was up-regulated in MII oocytes, was targeted by both *MIR542* and *MIR320a*. To visually assess the differentially expressed predicted miRNA target genes in CC and oocyte samples, we performed a supervised hierarchical clustering analysis (Fig. 4B).

Discussion

In this work we investigated the miRNA content of human MII oocytes and CCs. Analysis of the sequencing data from the two small RNA cDNA libraries indicates that overall the miRNA composition of the two cell types is different. Notably, the number of sequences

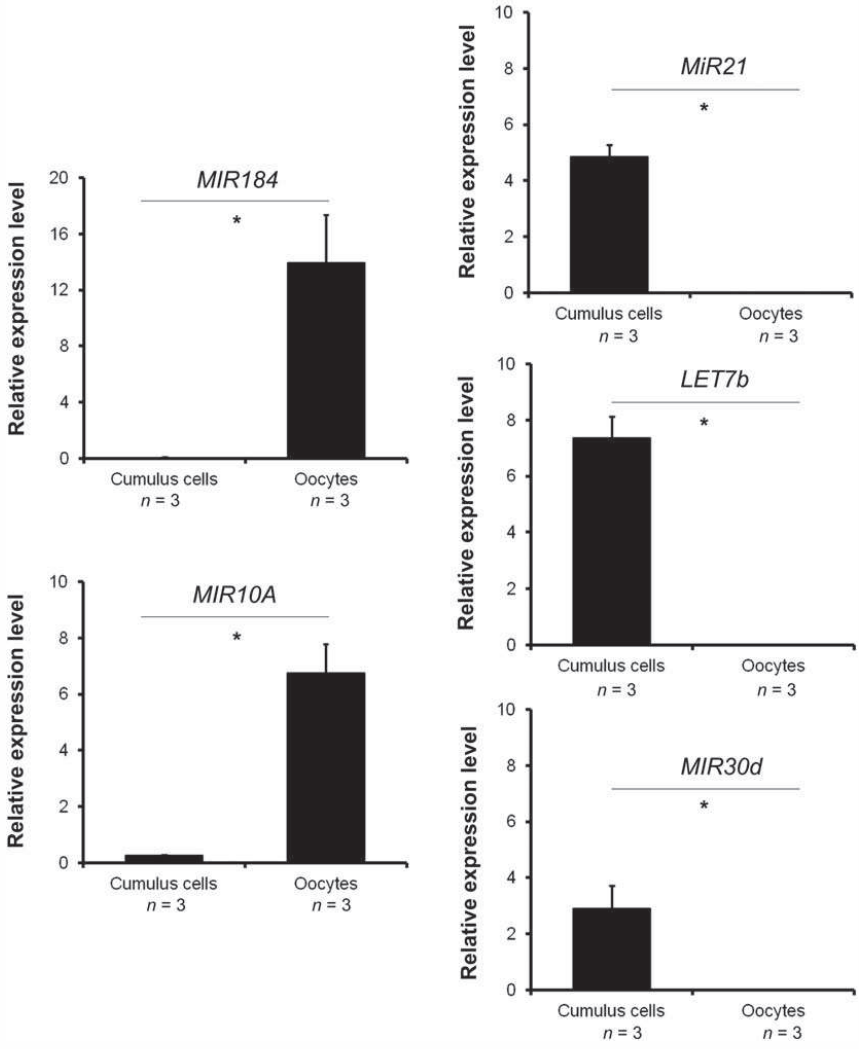

Figure 2 Relative expression levels of *MIR21*, *MIR30d*, *LET7b*, *MIR184* and *MIR10A* in MII oocytes and cumulus cells (CCs) by qRT–PCR. The transcript levels were calculated relative to the expression of RN *U6-1*. The mean ± SEM for each sample is presented in the bar graphs. Asterisk = $P < 0.01$.

corresponding to known miRNAs was higher in the CC library than in the mature MII oocyte library. This is consistent with the hypothesis that in germ cells, the number of expressed miRNAs is stage specific and decreases during development while the number of piRNAs increases (Malone *et al.*, 2009; Faunes *et al.*, 2012). Accordingly, the two size distribution peaks for the small RNA sequences of the MII oocyte library suggest that the transition of a major small RNA class from miRNA to piRNA might play a crucial role in the human oocyte–cumulus crosstalk, in line with previous findings (Girard *et al.*, 2006; Ohnishi *et al.*, 2010; Suh *et al.*, 2010; Tam *et al.*, 2008; Yang *et al.*, 2012a). Moreover, comparison of the miRNA expression profiling data and the list of target mRNAs that were differentially expressed in MII oocytes and CCs indicated that many genes that are up-regulated in MII oocytes are potential targets of CC miRNAs, thus suggesting that the oocyte–CC crosstalk might be mediated also via miRNAs. In addition, miRNAs have been reported to show a dynamic change during oocyte maturation in the mouse (Tang *et al.*, 2007), bovine (Tesfaye *et al.*, 2009; Mondou *et al.*, 2012) and human (Xu *et al.*, 2011).

Expression profiling of miRNA by deep sequencing indicated that *MIR100*, *MIR184* and *MIR10A* are specifically expressed in human MII oocytes. These miRNAs appear to be involved in the regulation of gene transcription, cell cycle and oocyte reprogramming. Interestingly, the comparison of our data on human oocytes with those reported for miRNA profiles in oocytes from other species (mouse, bovine and human) indicates that *MIR10* and *MIR100* are restricted to human and bovine oocytes (Tang *et al.*, 2007; Abdel El Naby *et al.*, 2013). It is worth noting that these miRNAs of interest are not affected by using MII oocytes that have failed to fertilize as source of material.

In human CCs, the *LET7* miRNA family is the most abundant miRNA cluster and *let-7b* is the most abundant individual miRNA. This supports previous miRNA expression profile studies that identified the *LET7* miRNA family as abundantly expressed in mouse and bovine ovaries

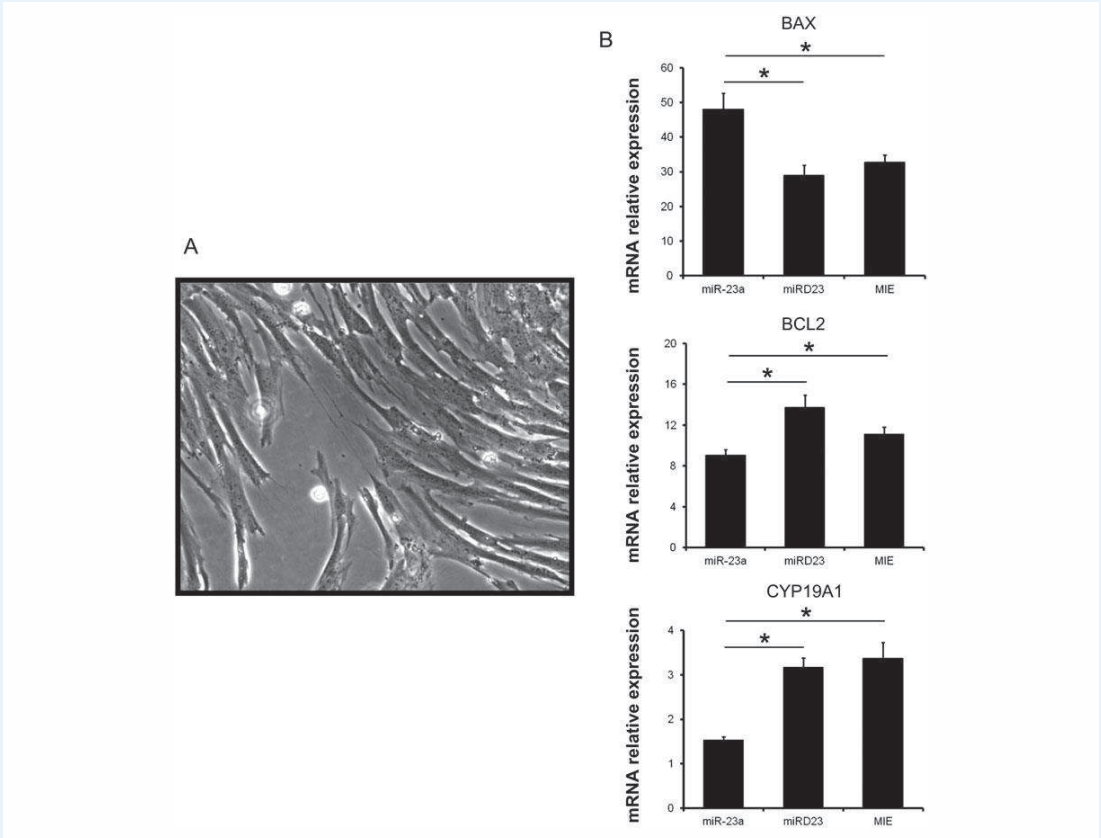

Figure 3 Biological effects of *MIR23a* in cultured CCs. (**A**) Phase contrast micrographs of primary CC cultures used as a model to test the functionality of *miR-23a*. (**B**) Primary CCs (P3) were transfected with the wild-type *MIR23a* locus, *MIRD23* (locus without *MIR23a*) or the MIE retroviral vector alone. 48 h after transfection, the expression of *BAX, BCL2* and *CYP19A1* was checked by qRT–PCR. Asterisk, $P \leq 0.05$. The transfection experiments were repeated three times and the qRT–PCR experiments were performed in triplicate.

(Reid *et al.*, 2008; Wyman *et al.*, 2009; Miles *et al.*, 2012) and in developing human ovary (Childs *et al.*, 2012). Additionaly, the comparison between our study on human CCs and previously published study on bovine CCs (Abdel El Naby *et al.*, 2013) reveals that *LET7b*, *LET7c LET7g MIR210* and *MIR125a* are expressed in both.

Globally, miRNAs that are abundant in CCs appear to be associated with the regulation of ECM and apoptosis. ECM formation through the production of ECM components by CCs is an important process that determines oocyte maturation and fertilization (Russell and Salustri, 2006; Dunning *et al.*, 2012). Analysis of the microarray data from individual CCs revealed that collagen genes (*COL4A1*, *COL4A5*, *COL3A1*, *COLA2*, *COL1A1*) are up-regulated in CCs compared with MII oocytes and are predicted to be *MIR29a* targets. Among the various biological functions of this miRNA, there is also the regulation of the expression of ECM components in different organs, including heart, lung, kidney and liver (van Rooij *et al.*, 2008; Jiang *et al.*, 2010; Cushing *et al.*, 2011). It is clear that the biological functions of *MIR29a* are complex, but the direct participation of this miRNA in regulating the expression of ECM components in human CCs needs further investigation.

CC apoptosis may compromise the oocyte developmental competence (Lee *et al.*, 2001) and elevated CC apoptosis has been associated with oocyte maturation delay and poor pregnancy outcome (Lee *et al.*, 2001; Host *et al.*, 2002). Here we show that the anti-apoptotic *BCL2*, *MCL1* and the pro-apoptotic *BAX*, *CASP3* and *CASP7* are up-regulated in CCs and are predicted targets of several CC miRNAs, suggesting a role for miRNAs in the regulation of apoptosis in COCs. Our findings also indicate that some miRNAs can target genes with opposite functions. For instance, *MIR29a* appeared to target both anti-apoptotic (*BCL2* and *MCL1*) and pro-apoptotic (*CASP7*) genes, suggesting that it may play roles in pathological conditions that might lead to ovarian failure. A recent study showed that *MIR23a* induces apoptosis in human GC (Yang *et al.*, 2012b). Similarly, we observed that after transient transfection of the whole *MIR23a* locus in primary cultures of CCs isolated from mature COCs, the mRNA expression of the anti-apoptotic

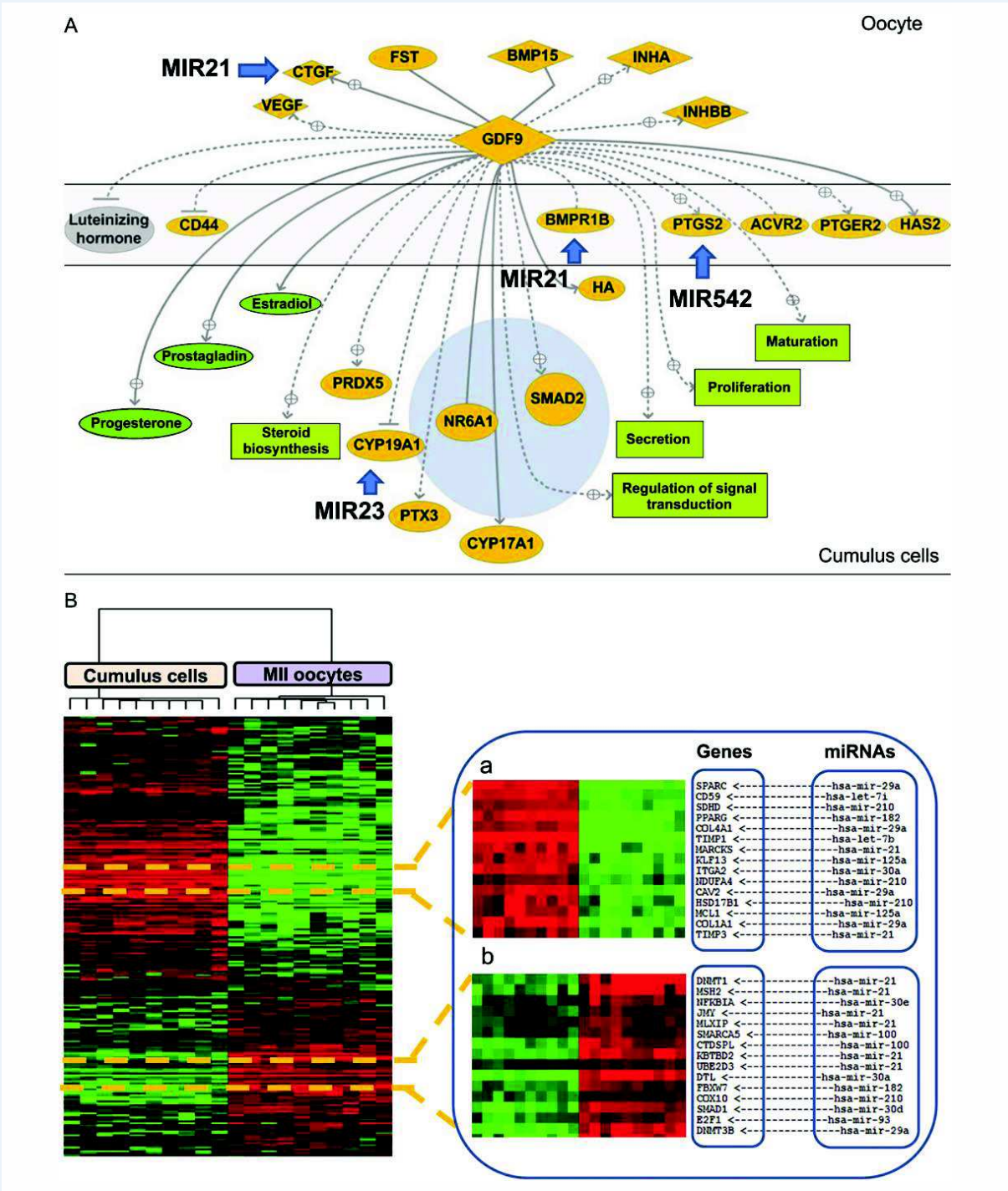

Figure 4 (**A**) Schematic representation of the components of the *GDF9* signaling pathway and targeting CC miRNAs. Some CC-specific genes known to be regulated by the oocyte-secreted paracrine factor (*GDF9*) (Elvin *et al.*, 1999), such as prostaglandin endoperoxide synthase 2 [*PTGS2* or cyclooxygenase-2 (*COX-2*)] and connective tissue growth factor (*CTGF*), are predicted targets of miRNAs that were identified in the CC small RNA cDNA library (*MIR542* and *MIR21*, respectively) (Adam *et al.*, 2012; Moore *et al.*, 2012). (**B**) Heat map representation of the 224 genes that are differentially expressed in MII oocytes and CCs and that are potential targets of the miRNAs identified by deep sequencing. (a) Cluster of targeted genes that are up-regulated in CCs but not MII oocytes. (b) Cluster of targeted genes that are highly expressed in MII oocytes but not CCs. Over-expression (red) and under-expression (green). Rows, genes; columns, profiled samples. The detailed list of the target genes and miRNAs is in Supplementary data, Table VI.

BCL2 was reduced and that of the pro-apoptotic *BAX* was increased in comparison with CC cultures transfected with the *MIRD23* construct or with control vector (MIE). Additionally, *MIR21*, which was one of the most abundant miRNAs in the CC library, has a critical role in maintaining the survival of GC in periovulatory follicles in response to luteinizing hormones and acts as an anti-apoptotic factor in cultured murine GC (Fiedler *et al.*, 2008; Carletti *et al.*, 2010; Hennebold, 2010). All these results suggest that both *MIR21* and *MIR23* play an important role in controlling transcripts that are involved in the ovulatory follicle apoptosis. Understanding the mechanisms through which the *MIR21*, *MIR23* and *MIR29a* affect CC apoptosis may help to explain their potential role in the pathogenesis of ovarian failure (Yang *et al.*, 2012b).

Finally, our study shows that several DNA methyltransferases (*DNMT*s) are up-regulated in MII oocytes are targeted by *MIR29a* and *MIR21* (Fabbri *et al.*, 2007; Zhang *et al.*, 2011), two miRNAs which were identified in the CC miRNA library. This suggests a CCs–oocyte miRNA trafficking possibly via gap junctions and points to novel functions for miRNAs in the COC crosstalk. Moreover, alteration of the *MIR21* or *MIR29a*-dependent regulation of *DNMT* expression could be one of the key molecular events leading to abnormal DNA methylation in oocytes and could be associated with a decrease in reproductive potential (Yue *et al.*, 2012).

Conclusion

This study provides the first characterization of the miRNA profile in human CCs and MII oocytes using a deep-sequencing approach combined with genome-wide gene expression arrays. The present findings suggest that miRNAs could play a role in the regulation of oocyte and CC cross-talk.

Supplementary data

Supplementary data are available at http://humrep.oxfordjournals.org/.

Acknowledgements

We thank the direction of Montpellier 1 University and of Montpellier University Hospital for their support. We thank Dr Rathore Moeez for technical support with *MIR23a* transfections and Dr Apparailly Florence and Dr Duroux-Richard Isabelle for excellent technical assistance and discussion.

Authors' roles

S.A. conceived, designed and performed the experiments, analyzed and interpreted the data and wrote the paper; T.A. and D.P. performed the experiments; D.H., NP., C.H.L., D.P., T.C. and H.D. contributed to the data analysis; O.A. contributed to data interpretation, paper redaction and manuscript revision. S.H. conceived the study, analyzed and interpreted the data, wrote the paper and gave final approval.

Funding

This work was supported in part by Ferring Pharmaceuticals A/S.

Conflict of interest

The authors of the study have no conflict of interest to report.

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- Article 2

Female aging alters expression of human cumulus cells genes that are essential for oocyte quality. **Al-Edani T**, Assou S, Ferrières A, Bringer Deutsch S, Gala A, Lecellier C, Aït-Ahmed O, Hamamah S, (2014). *Biomed Res Int., 964614.*

Hindawi Publishing Corporation
BioMed Research International
Volume 2014, Article ID 964614, 10 pages
http://dx.doi.org/10.1155/2014/964614

Research Article

Female Aging Alters Expression of Human Cumulus Cells Genes that Are Essential for Oocyte Quality

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Received 2 July 2014; Revised 15 July 2014; Accepted 17 July 2014; Published 3 September 2014

Academic Editor: Calvin Yu-Chian Chen

Impact of female aging is an important issue in human reproduction. There was a need for an extensive analysis of age impact on transcriptome profile of cumulus cells (CCs) to link oocyte quality and developmental potential with patient's age. CCs from patients of three age groups were analyzed individually using microarrays. RT-qPCR validation was performed on independent CC cohorts. We focused here on pathways affected by aging in CCs that may explain the decline of oocyte quality with age. In CCs collected from patients >37 years, angiogenic genes including *ANGPTL4*, *LEPR*, *TGFBR3*, and *FGF2* were significantly overexpressed compared to patients of the two younger groups. In contrast genes implicated in TGF-β signaling pathway such as *AMH*, *TGFB1*, inhibin, and activin receptor were underexpressed. CCs from patients whose ages are between 31 and 36 years showed an overexpression of genes related to insulin signaling pathway such as *IGFBP3*, *PIK3R1*, and *IGFBP5*. A bioinformatic analysis was performed to identify the microRNAs that are potential regulators of the differentially expressed genes of the study. It revealed that the pathways impacted by age were potential targets of specific miRNAs previously identified in our CCs small RNAs sequencing.

1. Introduction

In developing countries, the first baby is conceived with a delay that keeps increasing. With aging there is natural decline in female fertility, which raises crucial issues for the society. The fertility decline is slow and steady in 30 to 35 years old women. However, this decline accelerates past 35 years due to the decrease in oocyte quality and ovarian reserve [1, 2]. Therefore female age is crucial and oocyte aging is a common cause of assisted reproduction technology failures [3]. MII oocyte stores large quantities of mRNA and proteins and contains a high number of mitochondria [4, 5]. Oocytes from women with an advanced reproductive age may have an increase of oxidative stress with consequences on mitochondrial DNA (mtDNA) integrity, resulting in mitochondrial dysfunction [6, 7]. Interestingly transcriptome profiles showed a substantial difference between younger and older human oocytes [8]. Moreover the increase of aneuploidy due to aging is well documented. Indeed, the link between female age and oocyte aneuploidy prevalence was extensively studied [9]. However both intrinsic (oocyte) and/or extrinsic (follicular) factors may be involved in the oocyte quality decline. The ovarian follicular microenvironment, mediated through cumulus cells (CCs), is crucial for the development of competent oocytes [10]. The CCs are in physical contact with the oocyte; together they form the cumulus-oocyte complex (COC) and undergo a cross-talk [11]. The oocyte controls the differentiation and expansion of CCs, which in turn are responsible for the metabolism of the glucose and pyruvate used for energy production in

the oocyte [12]. An aged follicular microenvironment could impact oocytes and leave a characteristic transcriptional footprint in the surrounding CCs. Indeed, the use of human CC gene expression has proved powerful as a noninvasive approach to predict oocyte quality and developmental potential [13–16]. The analysis of gene expression in human CCs in relation to female age is based on the same rationale [17–19]. However, with the exception of one proteomic analysis [17], no high throughput study based on gene expression profile in relation to female age was performed on cumulus cells. Our hypothesis here is based on the assumption that female age may have a wide impact on gene expression and may specifically affect pathways that are critical for oocyte quality and development. The purposes of this study were (i) to thoroughly evaluate impact of maternal age on gene expression profiles using individual CCs isolated from the periovulatory follicles of three age categories of patients, (ii) to characterize the pathways that were significantly affected by female aging, and (iii) to identify their miRNAs regulators.

2. Materials and Methods

2.1. Sample Characterization and Collection. The Review Board of the Institute of Research in Biotherapy approved this project. All patients provided their written informed consent for the use of CC samples for research.

CC samples were collected from patients who participated to the multicentric trial previously described [20] and from Montpellier ART centre. Patients were stimulated with a combination of GnRH antagonist protocol with recombinant FSH or with HP-hMG before undergoing intracytoplasmic sperm injection (ICSI) procedure for male infertility. Cumulus oocyte complexes (COCs) were recovered under ultrasound echo-guidance 36 h after human Chorionic Gonadotrophin (5,000 UI, hCG) administration. CCs were separated mechanically from the corresponding oocyte as previously described [14]. For microarray 28 individual CC samples obtained from 16 patients were classified into three age groups: <30 years ($CC_{younger}$), 31–34 years (CC_{median}), and >37 years (CC_{older}). The qRT-PCR analyses were performed on 15 independent CCs from the above groups and 4 CCs from a 35-36 additional group.

2.2. RNA Extraction and Microarray Processing. CCs were frozen at −80°C in RLT buffer before RNA extraction. Then the RNeasy Micro kit (ref: 74004; Qiagen) was used to extract total RNA from each CC sample, according to the manufacturers' recommended protocols. The quantity and purity of the total RNAs were determined by using a NanoDrop ND-1000 spectrophotometer (NanoDrop ND-Thermo Fisher Scientific, Wilmington, DE, USA) and their integrity determined by using the Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, http://www.agilent.com). All RNA samples were stored at −80°C. Microarray experiments were performed on the microarray platform of Institute of Research in Biotherapy at the Montpellier University Hospital. The Affymetrix 3′ IVT express protocol (reference 901229) was used as previously described [20].

2.3. Microarray Data Analysis. After image processing with the Affymetrix GeneChip Operating 1.4 software, the CEL files were analyzed using the Affymetrix Expression Console Software v1.3.1 and normalized with the MAS5.0 algorithm by scaling each array to a target value of 100 using the global scaling method. This algorithm also determines whether a gene is expressed with a defined "detection call." This "call" can either be "present" (when the perfect match probes are significantly more hybridized than the mismatch probes, $P < 0.04$), "marginal" ($0.04 < P < 0.06$), or "absent" ($P > 0.06$). Gene annotation was performed using NetAffx (http://www.affymetrix.com; March 2009). A first selection using the detection call (present in at least seven samples) and variation coefficient (≥40%) of CC samples identified 9,802 transcripts. Then, to compare the three groups of CCs according to maternal age, a Significance Analysis of Microarrays-Multiclass (SAM-M) (http://statweb.stanford.edu/~tibs/SAM/) was used. SAM-M handed the significantly expressed genes with a q-value <5% in the three age categories. CLUSTER and TREEVIEW software packages were used for the hierarchical clustering analysis. SPSS 12.0 (SPSS, Chicago, IL) software was used for box-and-whisker plots representation of expression levels of specific genes. The miRNA target predictions were performed with GeneGo MetaCore analysis software (St. Joseph, MI). Ingenuity Pathway Analysis software and DAVID (http://david.abcc.ncifcrf.gov/) were used for functional annotation.

2.4. Quantitative RT-PCR. Reverse transcription (RT) was performed as recommended by the manufacturer (Invitrogen) with 150 ng of RNA in a 20 μL reaction volume that included Superscript II (ref. 18064-014, Invitrogen), oligo-dT primer, dNTP mixture, MgCl2, and RNase inhibitor. Quantitative PCR was performed using the SYBR Green I Master kit (Roche Diagnostics, Mannheim, Germany) with 2 μL of 1/20 dilution of the RT reaction product and 0.5 mM primer (SIGMA Genosys) in a total volume of 10 μL. The amplification was run in a LightCycler 480 apparatus as follows: after the denaturation step for 10 min at 95°C, cycling conditions were 10 s at 95°C, 30 s at 65°C and 1 s at 72°C for 45 cycles. Gene expression levels were normalized to the housekeeping gene Glyceraldehyde 3-Phosphate Dehydrogenase (*GAPDH*) using the following formula $100/2^{\Delta\Delta Ct}$ where $\Delta\Delta Ct = \Delta Ct_{unknown} - \Delta Ct_{positive\ control}$. The primer sequences are shown in (see Table SI in Supplementary Material available online at http://dx.doi.org/10.1155/2014/964614).

2.5. Statistical Analysis. Statistical analysis was performed with the GraphPad InStat 3 software. For qRT-PCR, the Kruskal-Wallis nonparametric test was used. The differences among the groups were considered significant when the P value is <0.05.

3. Results

3.1. Gene Expression Profiles of CCs according to Female Age. In order to gain insight into the molecular basis of age

impact on COCs, we analyzed the transcriptomes of CCs from women with different age categories. A first selection based on the detection call and variation coefficient of all the CC samples from aged and young patients delineated 9,802 transcripts. Then, using SAM-M and after having discarded 35 genes that we previously showed to be affected by the COS protocols [20], we identified a total of 2,186 transcripts (corresponding to 1,874 genes) with a q-value <5% that significantly distinguished the three CC groups according to female age (Supplementary Table SII). The analysis of the transcriptome data revealed a characteristic molecular signature for each one of the three age categories (Figure 1). The expression patterns of the genes that best represent these categories are illustrated in the box-plots (Figure 1(a)). In $CC_{younger}$ group, overexpression was observed for inflammatory response genes such as *B4GALT1, SERPINA1, C1S, IL18R1, FN1,* and *OSMR*. The CC_{median} group revealed overexpression of genes involved in insulin signaling pathway, the most representative being *IGFBP3, IGFBP5* and *PIK3R1*. Finally the CC_{older} group was significantly enriched with genes that are important for angiogenesis such as *ANGPTL4, LEPR, TGFBR3, VEGFC, FGF2* and *NR2F2*. In addition, a list of 20 genes with the highest contrast and lowest q-value according to SAM-M, were chosen for each category to perform the hierarchical clustering (Supplementary Table SIII). Interestingly, CC_{older} samples distantly located from the $CC_{younger}$ and CC_{median} samples (Figure 1(b)).

3.2. Validation of Gene Expression by Quantitative RT-PCR. Nine differentially expressed genes were selected for validation on the basis of relevant functional annotations. Hence, three genes involved in the inflammatory process (*B4GALT1, SERPINA1,* and *C1S*), three genes of the insulin signaling (*IGFBP3*, *IGFBP5,* and *PIK3R1*) and three genes of the angiogenesis process (*ANGPTL4*, *LEPR,* and *TGFBR3*) were chosen for qRT-PCR validation. Analysis of the qRT-PCR data on independent cohorts of CCs indicated that all the selected genes were differentially expressed in the three age categories and in agreement with the microarray findings (Figure 2). Using qRT-PCR we aimed to test the expression level of the above genes in individual CCs from 35 and 36 old patients. These CCs clearly displayed an expression pattern similar to the CC_{median} age category (Figure SI) suggesting that the switch for these genes occurs after the age of 36.

3.3. Deregulation in CC_{older} Genes that Are Essential for the Oocyte Quality and Competence. Many biological pathways were reported to be crucial for their impact on the oocyte development. They include transforming growth factor β (TGF-β) signaling, steroidogenesis and metabolic pathways. Interestingly the key members of these pathways displayed significant changes in their gene expression (Table SII). As shown in Figure 3(a), many genes of the TGF-β signaling pathway were underexpressed in CC_{older} compared with $CC_{younger}$ and CC_{median}, including *AMH* (Anti-Mullerien Hormone), *TGFB1*, inhibin (*INHA*) and activin receptor (*ACVR2B*). In contrast overexpression was observed in CC_{older} for several genes that are involved in steroidogenesis and fatty acid metabolism (*HSD17B1, HSD17B6, NSDHL, SRA1, CYP19A1, PPARA*), glucose metabolism (*ALG13, GLT8D3*) and glucose transporters (*SLC2A3*, *SLC2A1*, *SLC2A13*, *SLC2A8*). It is noteworthy that several genes that play an essential role in the cumulus-oocyte dialog (*INHA*, *CD200* and *IL6ST*) were downregulated in CC_{older} (Figure 3(b)). Moreover, CC_{older} may be distinguished from the two younger age categories by a down-regulation of genes that are essential for genome integrity, in particular *MSRB3, UCHL5IP, POLH, OBFC2B,* and *CHAF1A* that are essential for antioxidative and DNA repair functions.

3.4. Potential miRNA Regulators of the Differentially Expressed Genes of the Study. Using the GenGo Metacore software, we first aimed to identify which miRNAs regulate the genes that were overexpressed in each of the three age categories, $CC_{younger}$, CC_{median} and CC_{older} (Figure 4(a)). We identified altogether 286 miRNAs that are putative regulators of the differentially expressed genes identified in this study, among which 176 are common putative regulators of the genes overexpressed in the three age categories, 71 for the genes whose expression was higher in $CC_{younger}$ and CC_{median}. Only one miRNA was shared by CC_{median} and CC_{older} categories specifically; similarly genes overexpressed in $CC_{younger}$ and CC_{older} had one specific miRNA in common. Interestingly this analysis also discriminates the CC_{older} from $CC_{younger}$ and CC_{median}, which may be considered as a super-group with common features. Some miRNAs were specific for one of the three age categories. Thirty-three miRNAs were identified as putative regulators of the genes overexpressed in CC_{older}, one for the $CC_{younger}$ and 3 for the CC_{median} categories (for the comprehensive lists, see Supplementary Table SIV). Among all the miRNAs retrieved by GenGo, 87% were identified by sequencing in CCs [21]. The fact that only the differentially expressed genes were submitted to GenGO may account for the missing 13%. There is another discrepancy between the list of the potential regulators and the miRNAs actually present in the CCs as identified in our previous work [21]. It is illustrated in Figure 4(a) for the two categories that stand out in the present study, namely the CC_{youger}-CC_{median} super-group (71) on the one hand and the CC_{older} (33) on the other hand. Among these potential miRNA regulators, only 6 are actually expressed in CCs: *MIR425*, *MIR744*, *MIR146b*, *Let-7d* for the CC_{youger}-CC_{median} super group and *MIR202, Let-7e* for the CC_{older}. This discrepancy might reflect a tissue specific expression of miRNAs. Interestingly *MIR202* is a potential regulator of the hyaluronan synthase-encoding gene *HAS2* that is related to aging and angiogenesis [22] and *MIR744* is a *TGFB1* validated regulator [23]. The largest set of miRNAs retrieved by GenGo was common to the three age categories (176). This set was crossed with those effectively expressed in CCs [21], resulting in a list of 22 miRNAs. We were interested in those that regulate significant gene members of the pathways and processes impacted by female age and that were also experimentally validated. The results of this analysis are shown in Figure 4(b). None fulfills these criteria for the validated genes of the inflammatory process overexpressed in the $CC_{younger}$. In CC_{median},*IGFBP3*,

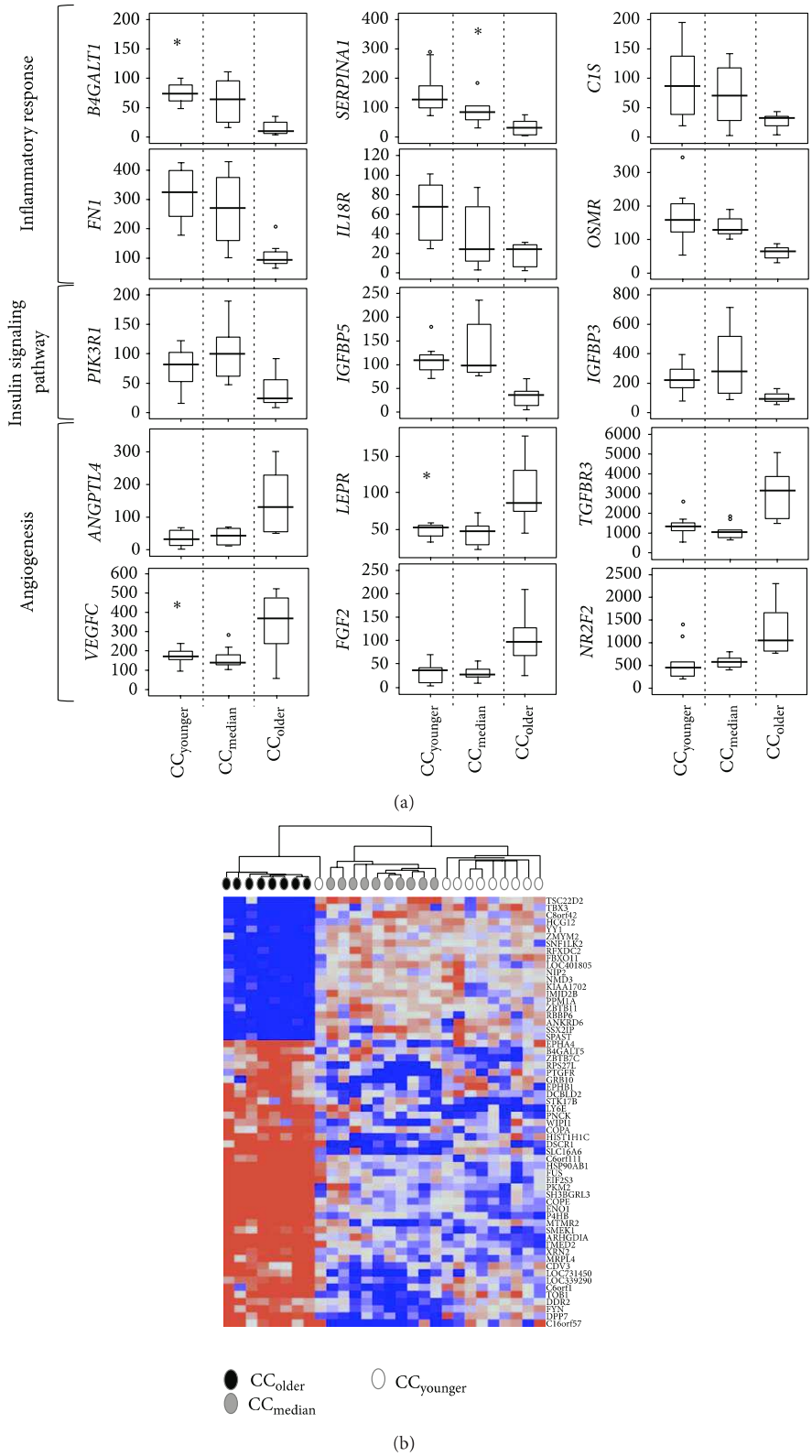


FIGURE 1: (a) Expression of cumulus cells genes according to female age. Box-and-whisker plots that represent expression of genes implicated in different biological processes and signaling pathways in the three female age categories, $CC_{younger}$, CC_{median}, and CC_{older}. The signal intensity of each gene is shown on the y axis as arbitrary units determined by the Affymetrix GCOS software. (b) Heat map and cluster dendograms of differentially expressed genes. Hierarchical clustering is shown for 20 genes with the highest expression level in each of the 3 age categories of individual CCs. Overexpressed and underexpressed genes were marked in blue and pink, respectively. The three age categories are shown in white for $CC_{younger}$, grey for CC_{median}, and black for CC_{older}.

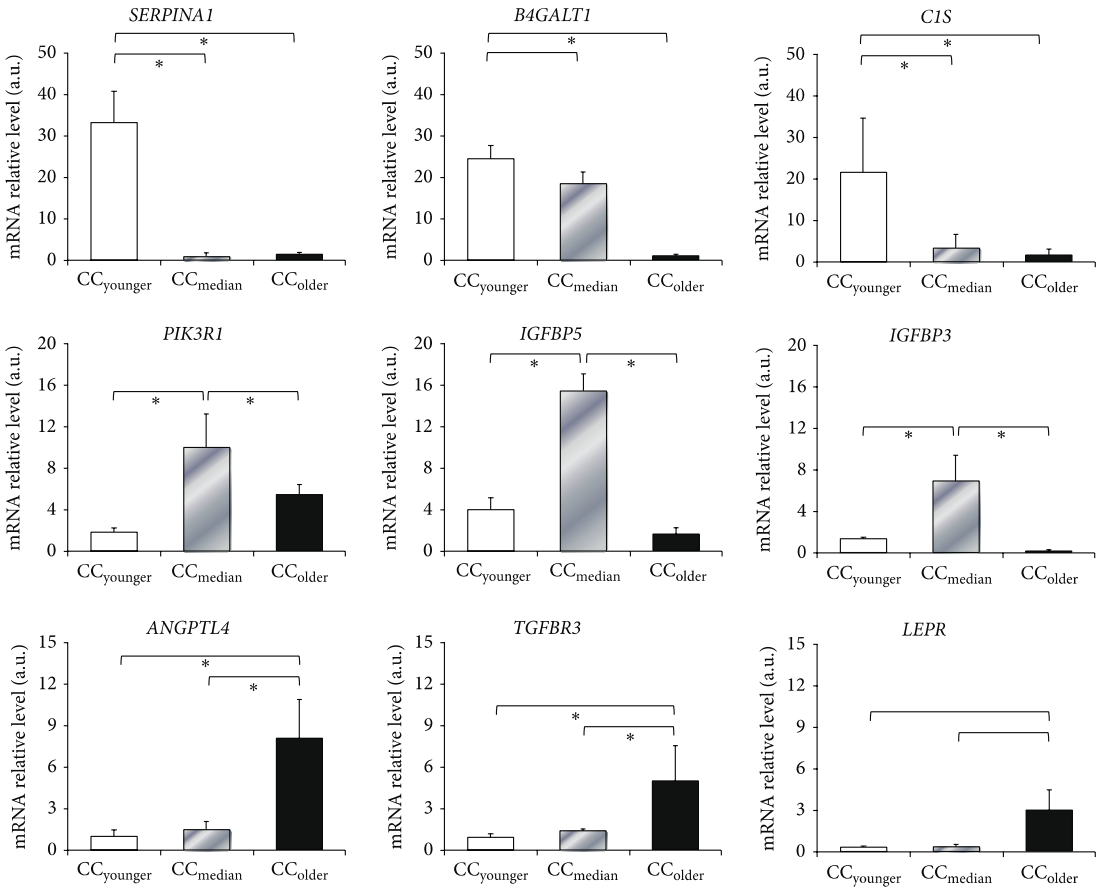


Figure 2: Validation by qRT-PCR of some gene members of key pathways that are differentially expressed in the three age categories. This figure shows the mRNA relative abundance of three genes implicated in inflammatory response (*SERPINA1*, *B4GALT1*, and *C1S*), three genes in insulin signaling (*PIK3R*, *IGFBP3*, and *IGFBP5*), and three genes in angiogenesis process (*ANGPTL4*, *TGFBR3*, and *LEPR*). The signal intensity for each gene is shown on the y-axis in arbitrary units determined by RT-qPCR analysis. * indicates a significant difference of gene expression between CCs categories ($^{*}P < 0.05$). Results were presented as the mean ± SEM. $CC_{younger}$ (white, age: <30 years), CC_{median} (grey, age: 31–34 years), and CC_{older} (Black, age >37 years).

and *IGFBP5* of the insulin-signaling pathway are targets of *MIR210* and *MIR140*, respectively. Finally in CC_{older}, genes implicated in angiogenesis *LEPR* and TGFBR3 are *MIR21* targets whereas *FGF2* is targeted by *MIR424*. For more details see Table SV.

4. Discussion

Acquisition of oocyte competence is a gradual and complex process, which depends on the follicular microenvironment. Within this microenvironment, the bidirectional communication between the CCs and the oocyte plays a crucial role. Therefore gene expression in CCs mirrors the oocyte physiology. In order to gain insight into the mechanisms that underlie oocyte quality decline with age, we first investigated the transcriptome profiles in CCs from women of three age categories. Our objective was to identify molecular signatures characteristic of each age category and investigate their biological relevance to oocyte quality. DNA microarray analysis revealed a significantly distinct molecular signature of 1,874 genes among the three age groups, suggesting a wide impact of female age on the CC gene-expression profile. It is noteworthy that the inflammatory genes emerged in the $CC_{younger}$ group such as *IL18R1*, *IL1R1*, *IL1R2*, *SERPINA1*, and *B4GALT1*. Inflammatory reaction is known to induce ovulation through infiltration of leukocytes into the area surrounding the follicle [24]. Cytokines are important in the regulation of ovarian function and oocyte quality [25]. On the other hand interleukins *IL18* and *IL1β* were reported to be present in floating granulosa cells of human preovulatory follicles [26]. CC_{median} group may be characterized by an overexpression of gene members of the "insulin-signaling pathway", such as *IGFBP3* and *IGFBP5* whereas *INSR* was overexpressed in both the CC_{median} and CC_{older} groups. Several studies have shown that insulin and *IGF* system play an important role in folliculogenesis [27–29] and in oocyte maturation [30]. IGF-binding proteins (*IGFBPs*)

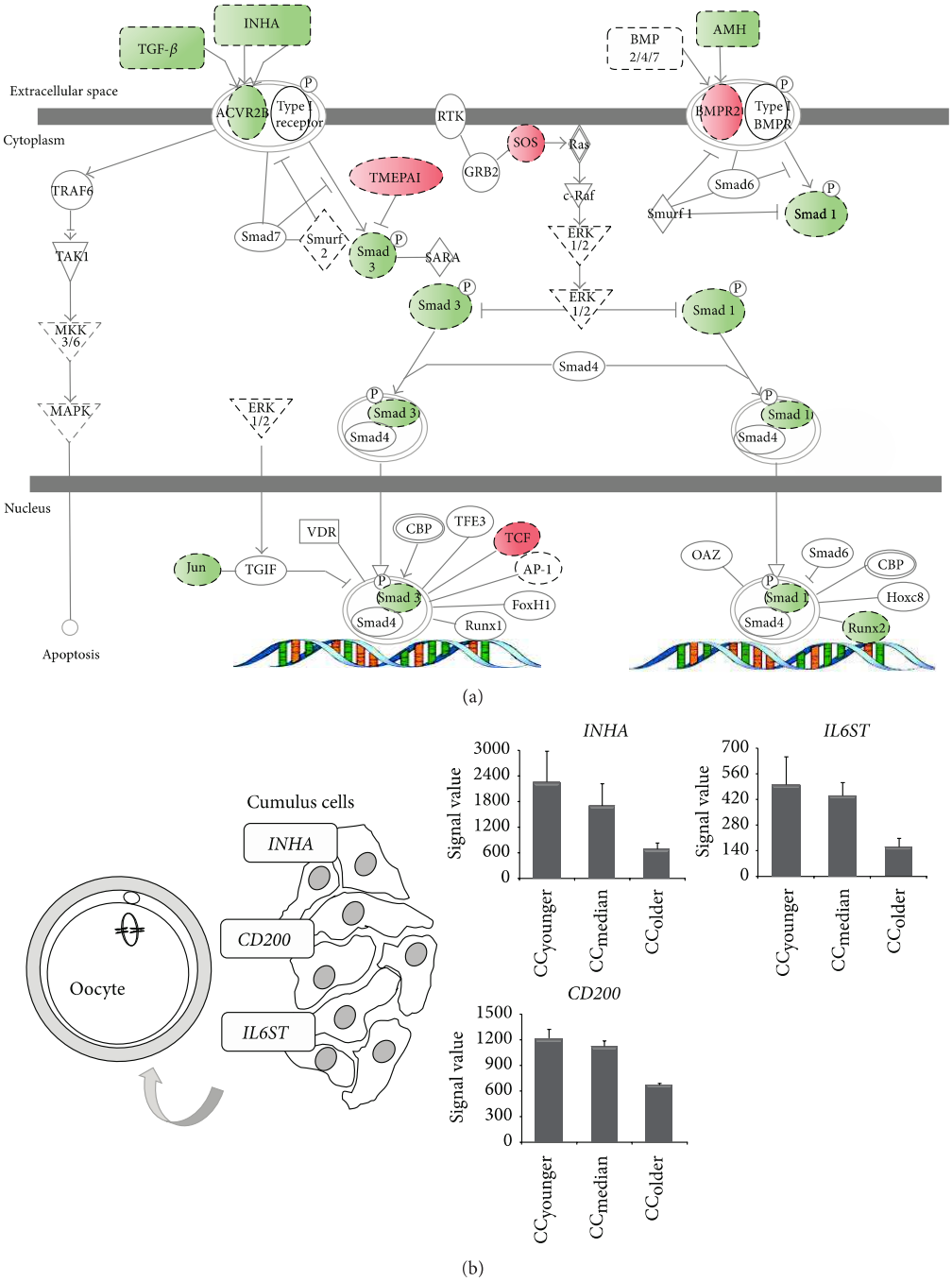
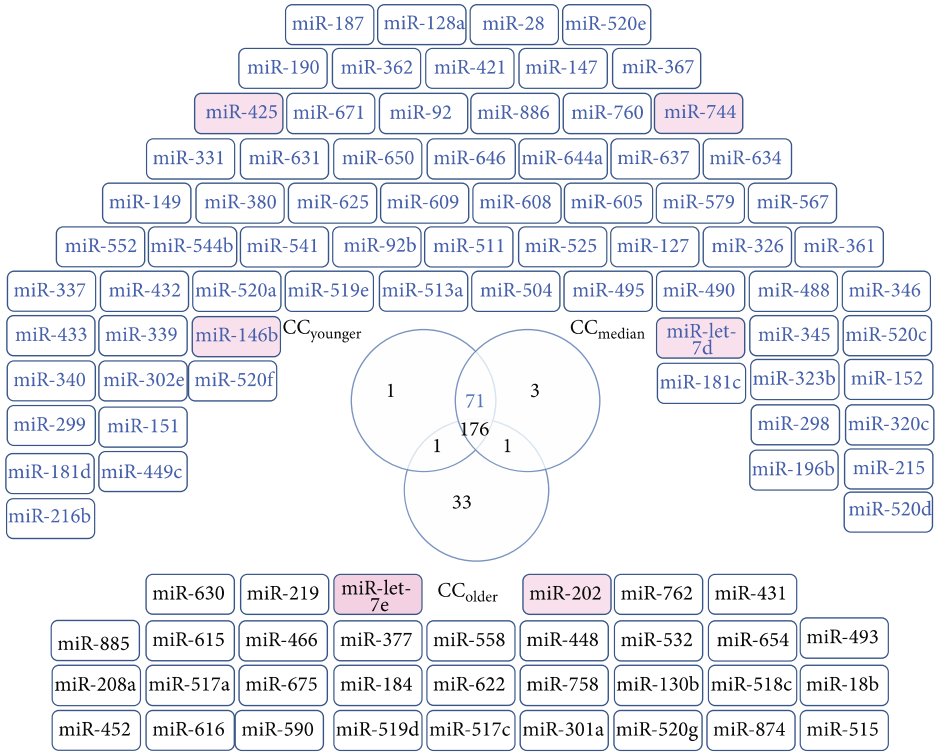
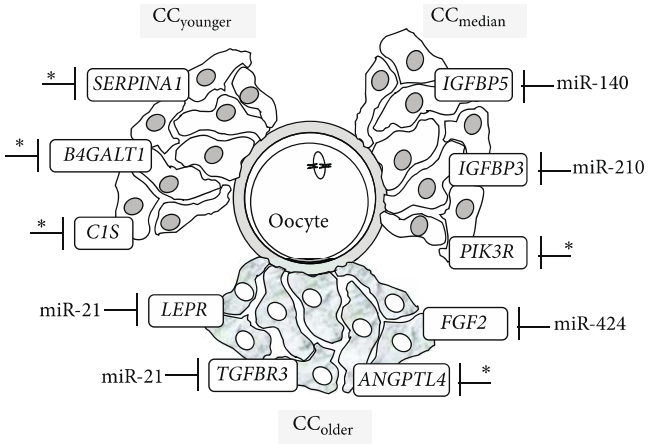


FIGURE 3: (a) TGF-β signaling pathway was deregulated in older CCs. The Ingenuity Pathway Analysis software was used to analyze impact of maternal age on TGF-β signaling. Downregulated genes in older CCs are shown in green and upregulated ones in red. Uncolored genes were not differentially expressed by our analysis but were integrated into the computationally generated networks on the basis of the evidence stored in the IPA knowledge memory indicating a relevance to this network. A plain line indicates direct interactions, a dashed line indicates indirect interactions, a line without arrowhead indicates binding only, a line finishing with a vertical line indicates inhibition, and a line with an arrowhead indicates "acts on." (b) Schematic representation of genes upregulated in $CC_{younger}$ and CC_{median} and downregulated in CC_{older} that are involved in cumulus-oocyte complex and oocyte development. Histograms show signal values of genes (*INHA*, *CD200*, and *IL6ST*) that are differentially expressed between age categories. Gene expression is measured by pan-genomic HG-U133 Plus 2.0 Affymetrix oligonucleotides microarrays, and the signal intensity for each gene is shown on the y-axis as arbitrary units determined by the GCOS 1.2 software (Affymetrix).



(a)



Gene target of miRNA	Name of gene	MiRNA	Effect	References (PMID)
IGFBP5	Insulin-like growth factor binding protein 5	miR-140	IGFBP5 is a direct target of miR-140	19948051
IGFBP3	Insulin-like growth factor binding protein 3	miR-210	IGFBP3 is validated miR-210 target	23028679
LEPR	Leptin receptor	miR-21	miR-21 target LepR in primary human keratinocytes	22773832
TGFBR3	Transforming growth factor, β receptor III	miR-21	TGFβRIII 3′-UTR containing the conserved miR-21 binding sites was validated by luciferase reporter assays	22960625
FGF2	Fibroblast growth factor 2	miR-424	FGF2 is described as experimentally validated miR-424 target	22965126
SERPINA1	Serpin peptidase inhibitor, clade A (α-1 antiproteinase, antitrypsin), member1			
B4GALT1	UDP-Gal: βGlcNAc β 1,4-galactosyltransferase, polypeptide 1			
C1S	Complement component 1, s subcomponent			
PIK3R	Phosphoinositide-3-kinase, regulatory subunit 1			
ANGPTL4	Angiopoietin-like 4			

(b)

FIGURE 4: (a) Venn diagram representing the number of miRNAs retrieved from the GenGo analysis. The genes overexpressed in each age category were submitted to GenGo to identify their potential miRNA regulators. 249 miRNAs were retrieved for the $CC_{younger}$ group, 251 for the CC_{median}, and 211 for the CC_{older}. The Venn diagram drawn after these lists shows that the majority is common to the three age categories. The miRNAs that are detected in the cumulus cells by using deep-sequencing approach [21] are shown in pink. (b) Schematic representation of some of the validated genes of the three pathways and processes discussed in this work and their miRNA regulators. Only the miRNAs that were found in the CCs small RNA sequencing and reported in the literature to be experimentally validated were represented. * indicates the validated genes with no miRNA regulator that meets these criteria.

that modulate interactions of *IGFs* with *IGF* and insulin receptors [31] have also an antiangiogenic activity [32–34]. Therefore, overexpression of *IGFBPs* in CC_{median} may be to modulate angiogenesis and maintain a balance. Last, the CC_{older} group is precisely characterized by an upregulation of genes associated with angiogenesis (*ANGPTL4, LEPR, TGFBR3, VEGFC, FGF2* and *NR2F2*). Angiogenesis plays a critical role in the late stages of folliculogenesis by providing nutrients and oxygen to the growing follicles. However, it may be associated with pathology and induced by microenvironmental factors like hypoxia. In this context, the follicular cells synthesize several angiogenic factors [26, 35, 36], among

which the vascular endothelial growth factor C (*VEGFC*) and angiopoietin-like 4 (*ANGPTL4*), which are induced in response to hypoxic stimuli [37–39]. So, the overexpression of angiogenic factors and hypoxia-inducible protein 2 (*HIG2*) in the CC_{older} group could be caused by insufficiency of oxygen. Similarly *VEGF* that is shown to increase in follicular fluid with age could be enhanced by hypoxia in old follicles [40, 41]. Most interestingly oocytes from hypoxic follicles have disorganized meiotic spindles [42]. These observations added to the reported increase of aneuploidy with female aging [43] may be revisited in light of our results. Hypoxia might be one of the consequences of aging, which in turn would affect chromosome segregation. Adaptive changes to oxygen availability are critical for cell survival and tissue homeostasis. Therefore, augmentation of angiogenesis in the CC_{older} group may be a compensatory process to modulate the deleterious impact of hypoxia. Similarly the upregulation of genes that encode metabolic enzymes (*HSD17B*, *CYP19A1*, *ALG13*, and *GLT8D3*) and glucose transporters (*SLC2A3*, *SLC2A1*, *SLC2A13*, *SLC2A8*) in the CC_{older} group could reflect a compensatory mechanism to increase energy production. These results are consistent with the observations reported recently [17, 19]. Indeed, the energy supplied by the CCs is known to be required for oocyte quality [44, 45]. Some members of the *TGF-β* superfamily, which are crucial to processes that govern follicle development and oocyte maturation [46], were underexpressed in the CC_{older} group such as *AMH* (Anti-Mullerien Hormone), *TGFB1*, inhibin (*INHA*), and activin receptor (*ACVR2B*). Interestingly *AMH* is produced by early primary follicles and its mRNA level is known to decrease with age. Therefore, it represents an early marker of ovarian follicle growth and a reliable marker of ovarian reserve and oocyte quality [47–49].

Another important question we addressed concerns the regulation of the genes that stand out in our study. We focused on the bioinformatic analysis of miRNAs. MiRNAs are noncoding small RNAs (18–25 nucleotides), which regulate cellular genes through RNA degradation or translational inhibition [50, 51]. Not only miRNAs have been shown to regulate the aging process in different tissues and cells [52], but their importance is also well recognized in the control of human cumulus-oocyte crosstalk and ovarian function and aging [21, 53, 54]. Interestingly, *TGF-β* signaling is one of the most significant pathways targeted by miRNAs contained in the follicular fluid [55]. Moreover gene members of this pathway are direct targets of *MIR21* that is the most abundant miRNA in CCs [21]. The role of *MIR21* is essential in ovarian function toprevent apoptosis in mouse periovulatory granulosa cells both *in vivo* and *in vitro* [56]. Moreover, it promotes the follicular cell survival during ovulation and is upregulated during luteinization [57]. Interestingly, a recent work reports a correlation between *MIR21* abundance and women age; a significant decrease was observed in follicular fluid of older women [53]. In the current study, two angiogenic genes (*LEPR* and *TGFBR3*) were upregulated in CC_{older} where *MIR21* is the least abundant [53]. Finally, the process that is central to this study is angiogenesis that may be induced in response to hypoxia, a major issue in aging follicles. Interestingly, miRNAs play a critical role in the cellular response to hypoxia [58]. *MIR210* whose overexpressionin hypoxic conditions induces angiogenesis [59, 60] directly targets *IGFBP3,* an inhibitor of angiogenesis [32, 61]. Furthermore, *MIR424* that is downregulated in response to hypoxia in primary human trophoblasts [62] targets *FGF2,* an angiogenesis inducer [63, 64]. Taken together these data suggest that in aging follicles angiogenesis may be induced in response to hypoxia by the underexpression of *IGFBP3* and overexpression of *FGF2*.

5. Conclusion

The present study reports for the first time an extensive analysis of gene expression in cumulus cells in relation to female age. Specific molecular signatures were characterized for the three age categories. Our findings point to aging as a major player in processes and pathways that are of key biological importance for oocyte growth and genome integrity. Moreover the upregulation of angiogenic genes in CC_{older} is very informative on the way the follicle attempts to buffer the deleterious impact of aging associated hypoxia. In addition to the transcriptomes, the comprehensive characterization of the miRNA regulators of the genes impacted by female age represents a valuable resource for future investigations on the biology of aging oocyte.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Tamadir Al-Edani and Said Assou contributed equally to this work.

Acknowledgments

This work was supported by Ferring Pharmaceuticals A/S. The authors thank the direction of the Montpellier 1 University and University Hospital of Montpellier for their support. They thank all members of ART team for their assistance during this study.

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Conclusion and future prospects

I- Conclusion

Success of assisted reproduction technologies strongly depends on oocyte quality. Since the evidence of a dialogue between the cumulus cells and the oocyte was recognized, cumulus cells have been widely studied to predict oocyte competence and quality. These studies were mainly dedicated to the transcriptome analysis leaving post-transcriptional regulation of this close relationship within human cumulus-oocyte complex elusive. The first part of my thesis work was therefore dedicated to an analysis of the miRNAs profile in human cumulus cells in addition to MII oocyte. Another important issue in oocyte quality and competence is the age impact. In a second part of my thesis work, I analyzed the transcriptome of the cumulus cells in relation to female age and performed an *in silico* analysis of their miRNA regulators. For the first part of the study, we performed deep sequencing of the cumulus cells small RNAs. The sequencing data revealed 32 known miRNAs in the CCs while only three known miRNAs were identified in human MII oocyte (*MIR184, MIR100*, and *MIR10A*). This is in contrast to mature bovine oocytes, which have 51 highly expressed miRNAs (Abd El Naby et al., 2013). Interestingly, in mice the role of miRNAs in the oocyte seems to be limited. This is suggested by the non-significant impact on oocyte maturation of an oocyte-specific deletion of DGCR8, a key enzyme in miRNA biogenesis (Suh et al., 2010). In comparison, mouse granulosa miRNAs have a wide array of roles in the regulation of numerous functions such as proliferation, apoptosis, and steroidogenesis (Carletti et al., 2010; Dai et al., 2013; Yan et al., 2012; Yao et al., 2010). These evidences are in agreement with our results, as CC-miRNAs probably control many biological functions within human cumulus-oocyte complex (COC) by targeting genes involved in cell assembly and organization, development, cell death and survival. Additionally CC-miRNAs potentially target many up-regulated genes in MII oocytes including genes associated with chromatin remodeling, such as DNA methyltransferase *DNMT3B* and *DNMT3A* (targets of *MIR29a*), *DNMT1* (a target of *MIR21*), and *CDC25A* (cell division cycle 25 homolog A), a key regulator of oocyte meiosis, a target of *MIR21*, *MIR424* and *LET7b*. Other CC-miRNAs predictively target some CC-specific genes known to be regulated by the oocyte-secreted factor (GDF9) such as *PTGS2* (a target of *MIR542*), the connective tissue growth factor *CTGF* and bone morphogenetic protein receptor *BMPR1B* (targets of *MIR21*). GDF9 is well known to mediate the bidirectional communication (Gilchrist et al., 2008; Gilchrist et al., 2004). Thus, all these results suggest potential involvement of miRNAs in the oocyte-CCs crosstalk. This is supported by a recent study (Pan et al., 2015) that reports the regulation of oocyte maturation via oocyte-cumulus interaction by expression of miR-378 in porcine cumulus cells. Moreover, the expression of

miRNAs in bovine oocyte or CCs is affected by the presence or absence of each of these two cell types during maturation (Abd El Naby et al., 2013).

In the second part of my study, considering that few data were available on the impact of women age on gene expression in cumulus cells, we performed the transcriptomic analysis of human CCs according to three age categories. Indeed age is known to be a risk factor that influences oocyte quantity and quality. Microarray data revealed distinct gene expression profiles according to the age. The functional analysis of the differentially expressed genes showed involvement of these genes in biological processes and pathways such as angiogenesis, TGF-β and insulin signaling pathways, that play a crucial role in acquisition of oocyte developmental competence (Fraser, 2006; Knight and Glister, 2003; Kwintkiewicz and Giudice, 2009). The central concept of this study turns around the angiogenesis and increased expression of angiogenic genes in the older CCs that might occur in response to hypoxia (Basini et al., 2004). Hypoxia is a key player in angiogenesis (Niemi et al., 2014). It is noteworthy that the severe cases of hypoxia are associated with oocyte aneuploidies (Van Blerkom et al., 1997). Interestingly hypoxia is one of the factors leading to ovarian aging (Tatone et al., 2008). We were also interested in the putative miRNA regulators of the genes impacted by maternal age as microRNAs are implicated in many biological processes including aging (Chen et al., 2010). Potential miRNA regulators of genes implicated in aging impacted-pathways and processes have been identified. Interestingly *MIR21* that is most abundant in human CCs decreases with aging in follicular fluid (Diez-Fraile et al., 2014). In this study, *MIR21* is a putative regulator of two angiogenic genes that are overexpressed in older CCs (*LEPR* and *TGFBR3*). *MIR21* decrease with aging may occur because of hypoxia. Indeed hypoxia is known to induce changes in the expression of a number of miRNAs (Nallamshetty et al., 2013). It is mentionable that *MIR21* is known for being inducible by hypoxia (Liu et al., 2014). Other studies show that it may also be downregulated by hypoxia (Sayed et al., 2010) as well as *MIR424* and *MIR210* that target respectively *FGF2* angiogenic gene overexpressed in older CCs and *IGFBP3* antiangiogenic gene underexpressed in older CCs. Interestingly downregulation of *MIR424* and overexpression of *MIR210* are exerted in response to hypoxia (Dang and Myers, 2015; Mouillet et al., 2013). Thereby, our data suggest that hypoxia has a potential role in follicle aging and impacts both gene expression and predicted miRNA regulators in CCs.

II- Future Prospects

In assisted reproductive technology (ART), ovarian aging is a critical factor for a succesful pregnancy that leads to live birth. Thus, development of tests to assess ovarian aging such as quantification of follicles, hormones measurement, and genetic testing is a necessity (Li et al., 2012). However, these tests are insufficient to predict pregnancy success and do not provide reliable indicators likely to reduce the high risks related to maternal age. Genomic disorders (embryo aneuploidies) are mostly due to age-related deficiency in oocyte quality. Therefore, the chances of ART success decrease with advancing female age, at the same time of significant increase in the number of women who attempt to conceive and demand ART treatment between the ages of 36 and 44 years (Bentov et al., 2011). It is noteworthy that preimplantation genetic screening (PGS) technique has emerged for aneuploidy detection and normal embryo transfer by using fluorescence in situ hybridization (FISH) or comparative genomic hybridization (CGH) (Fragouli et al., 2006; Munne et al., 2005), but it is expensive and probably accompanied by risks. Thus, ongoing studies in this domain focus on a search for non-invasive biomarkers of CCs or GCs (Fragouli and Wells, 2012). Interestingly, miRNAs appear to have considerable potency as emerging diagnostic biomarkers for ovarian disease detection and for selection of healthy competent oocytes and embryos in the ART (McGinnis et al., 2015). Additionally, implication of the miRNAs in crucial pathways for follicle and oocyte maturation also leads to consider them as biomarkers of oocyte quality (Santonocito et al., 2014). As well as, age-associated differential levels of miRNAs in human follicular fluid (Diez-Fraile et al., 2014) could open the doors for the potential use of these molecules as noninvasive biomarkers in ovarian reserve or aging assessment. Further, some CC-miRNAs are candidates to investigate as novel circulating biomarkers for ovarian function and IVF outcome (Traver et al., 2014). Finally, this study presented hypoxia-related CC-miRNAs as predictive candidates for oocyte aging and/or aneuploidy. This represents a starting gate for future investigations.

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Appendix

FORMATIONS

Récapitulatif de participation aux Formations
Tamadir ALEDANI

Doctorat : Biologie Santé
Ecole Doctorale : Sciences Chimiques et Biologiques pour la Santé
Etablissement : Université de Montpellier
Date de la 1ère inscription en thèse : 1 octobre 2012 (0 A en)
Directeur de thèse : Samir HAMAMAH
Sujet de thèse : Expression des ARNm et des microARN dans les cellules de cumulus humains : impact de l'âge maternel

Formations suivies

Catégorie : Conférences

* Molecular signature of human cumulus cells reveals impact of female age on pathways that are crucial for oocyte development 2013 - 2014 European Society of Human Reproduction and Embryology (ESHRE) – Munich, Allemagne
10 heures enregistrées par : Sciences Chimiques et Biologiques pour la Santé.

Total du nombre d'heure pour la catégorie Conférences : *10 h*

Catégorie : Formations scientifiques

* Advanced Markov Modeling (11 février 2013) 2012 - 2013 Campus saint priest. Bât.1. salle 30
21 heures enregistrées par : I2S - Information, Structures, Systèmes.

* Programmation orientee objet : calculs numériques en statistique et probabilité pour la biologie (30 mars 2015) 2014 - 2015 salle TD 9.01 (RDC, bâtiment 9, campus Triolet)
30 heures enregistrées par : I2S - Information, Structures, Systèmes.

Total du nombre d'heure pour la catégorie Formations scientifiques : *51 h*

Catégorie : Journée Doctorale

* Impact de l'âge maternel sur le profile transcriptionnel des cellules du cumulus 2013 - 2014 l'ecole doctorale CBS2– Montpellier, France
5 heures enregistrées par : Sciences Chimiques et Biologiques pour la Santé.

Total du nombre d'heure pour la catégorie Journée Doctorale : *5 h*

Catégorie : Langues

* FLE - Français Langue Etrangère (24 septembre 2013) 2012 - 2013 Université Montpellier 2. SCEL Bâtiment 5
30 heures Note : A2 enregistrées par : Collège Doctoral Languedoc Roussillon.

Total du nombre d'heure pour la catégorie Langues : *30 h*

Catégorie : Méthodologie et outils de la thèse

* Dépôt électronique de la thèse : présentation générale et aspects juridiques (24 mars 2015) 2014 - 2015
3 heures enregistrées par : Collège Doctoral Languedoc Roussillon.

* Recherche et gestion de l'information : méthodes, outils, ressources en Santé/Sciences (18 février 2013) 2012 - 2013
14 heures enregistrées par : Collège Doctoral Languedoc Roussillon.

* Start with R (10 juin 2015) 2014 - 2015
18 heures enregistrées par : Collège Doctoral Languedoc Roussillon.

* Writing a scientific paper step by step (18 novembre 2013) 2012 - 2013 Université Montpellier 2
14 heures enregistrées par : Collège Doctoral Languedoc Roussillon.

Total du nombre d'heure pour la catégorie Méthodologie et outils de la thèse : *49 h*

Catégorie : Outil pour la poursuite de carrière

* La thèse dans le cadre d'un projet professionnel (22 février 2013) 2012 - 2013
8 heures enregistrées par : Collège Doctoral Languedoc Roussillon.

Total du nombre d'heure pour la catégorie Outil pour la poursuite de carrière : *8 h*

Catégorie : Ouverture scientifique et culturelle

* Bioinformatique et ontologies (22 mai 2013) 2012 - 2013 Université Montpellier 2, bâtiment 25
25 heures enregistrées par : Sciences Chimiques et Biologiques pour la Santé.

Total du nombre d'heures pour la catégorie Ouverture scientifique et culturelle : *25 h*

Total participation : 178 heures / 11 modules

PUBLICATIONS

Female aging alters expression of human cumulus cells genes that are essential for oocyte quality.
Tamadir Al-Edani, Said Assou, Alice Ferrières, Sophie Bringer Deutsch, Anna Gala, Charles Lecellier , Ounissa Aït-Ahmed, Samir Hamamah. *Biomed Res Int. 2014, 964614.*

Traver S, Assou S, Scalici E, Haouzi D, **Al-Edani T**, Belloc S, Hamamah S. Cell-free nucleic acids as non-invasive biomarkers of gynecological cancers, ovarian, endometrial and obstetric disorders and fetal aneuploidy. *Hum Reprod Update. 2014 Jun 27. pii: dmu031. [Epub ahead of print] Review.*

Assou S, **Al-Edani T**, Haouzi D, Philippe N, Lecellier CH, Piquemal D, Commes T, Aït-Ahmed O, Dechaud H, Hamamah S. MicroRNAs: new candidates for the regulation of the human cumulus-oocyte complex. *Hum Reprod. 2013, 28:3038-3049*.

COMMUNICATIONS

Molecular signature of human cumulus cells reveals impact of female age on pathways that are crucial for oocyte development.
Al Edani, T.; Assou, S.; Aït-Ahmed, O.; Dechaud, H.; Hamamah, S. ***Oral communication** at 30th annual international meeting of European Society of Human Reproduction and Embryology (ESHRE) – Munich, Germany, from 29 June to 2 July 2014.
***Oral communication presented by myself**

Impact of maternal age on the transcriptional profile of human cumulus cells.
Al Edani T., Assou S, Aït-Ahmed O, and Hamamah S. Poster presentation in 12ème Journée de l'ecole doctorale CBS2– Montpellier, 23 mai 2014.

MicroRNAs regulate expression of aged human cumulus cells genes that are essential for oocyte quality.
Al Edani T., Assou S, Aït-Ahmed O, and Hamamah S. Oral presentation at the annual international meeting of American Society for Reproduction Medicine (ASRM)- Honolulu, in October 18-22, 2014.

Maternal age affects angiogenic factors and insulin signaling pathway of human cumulus cells isolated from mature MII oocyte.
Al Edani, T., S. Assou, S. Traver, O. Aït-ahmed, H. Dechaud, S.Hamamah. Poster presentation at 29th annual international meeting of ESHRE – London, United Kingdom, from 7 to 10 July 2013.

Maternal age affects insulin signaling pathway and angiogenic factors of human cumulus cells: importance of MIR-21 and MIR-140.
Al Edani, T., Said Assou, Anna Gala, Ounissa Ait-Ahmed, Hervé Dechaud and Samir Hamamah. Oral presentation at the annual international meeting of ASRM- Boston in October 12-17, 2013.